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Effects of Nutrient Level and Growth Rate on Mutation and Conjugation Processes That Confer Antibiotic Resistance to E. coli

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EFFECTS OF NUTRIENT LEVEL AND GROWTH RATE ON THE MUTATION AND CONJUGATION PROCESSES THAT CONFER ANTIBIOTIC RESISTANCE TO *E. COLI*

by

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Antibiotic resistance genes (ARGs) may proliferate in surface water following the discharge of treated wastewater effluent. Wastewater effluent often contains both antibiotic residues, which may cause the emergence of ARGs in water microbiome through mutation, and ARG-carrying bacteria, which can transfer ARGs to water microbiome through conjugation. However, little is known about how environmental factors affect these processes.

The goal of this study is to determine the effects of nutrient level and growth rate on the proliferation of ARGs in surface water receiving wastewater effluent. Specifically, the project investigated how environmental factors may affect the conjugation frequency and mutation rate that confer ARGs to recipient cells, as well as the resistance level of the recipient cells after mutation and conjugation. Chemostat reactors were built to simulate surface water receiving wastewater effluent. Two *E. coli* strains, CV601 and J53, were used as donor and recipient cells, respectively. First, CV601 cells received plasmids from an actual wastewater effluent sample through filter mating and became resistant to tetracycline. Then, J53 cells (i.e., to simulate water microbes) were established in

chemostats before CV601 cells (i.e., to simulate wastewater microbes) were introduced. Two nutrient levels (1/3 and 1/10 Muller Hinton broth) and two growth rates (0.15 and 0.45 hr^{-1}) were tested.

Results show that regardless the nutrient level, the conjugation frequency was 10^{-2} and 10^{-6} for the high and low growth rate, respectively. The minimum inhibitory concentration (MIC) of the recipient cells increased from 2 to 64-128 mg/L after the conjugation tests. In addition, recipient cells grown under 0.45 hr^{-1} and 1/10 MHB showed MIC increased from 2 to 8 mg/L in mutation experiment. Whole genome sequencing verified the presence of a plasmid containing tetracycline resistance genes in both the donor and the recipient cells, and identified the emergence of tetracycline resistance genes in recipient cells following mutation.

This study generates quantitative information on the proliferation of ARGs in the microbes of surface water receiving wastewater effluent. This information will allow for better modeling and prediction of the risk associated with ARG proliferation in the environment.

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LIST OF ABBREVIATIONS

AMR	Antimicrobial resistance
ANOVA	Analysis of variance
ARG	Antibiotic resistance gene
BLAST	Basic Local Alignment Search Tool
CFU	Colony forming unit
HGT	Horizontal gene transfer
HPLC	High performance liquid chromatography
LB	Lysogeny broth
MGEs	Mobile genetic elements
MHB	Mueller Hinton broth
MIC	Minimum inhibitory concentration
PBS	Phosphate buffer saline
WHO	World Health Organization
WWTP	Wastewater treatment plant

LIST OF SYMBOLS

C	*E. coli* CV601
CW	*E. coli* CV601 after filter mating with a secondary effluent of a WWTP, donor cells in this study
J	*E. coli* J53, recipient cells in the study
M15	*E. coli* J53 grown under 0.15 hr^{-1} in the mutation experiment
M45	*E. coli* J53 grown under 0.45 hr^{-1} in the mutation experiment
T15	*E. coli* J53 grown under 0.15 hr^{-1}, transconjugants in the conjugation experiment
T45	*E. coli* J53 grown under 0.45 hr^{-1}, transconjugants in the conjugation experiment

CHAPTER 1. OVERVIEW

1.1 Antibiotic & Antibiotic Resistance Genes History

The era of antibiotics began in the 1940s where Sir Alexander Fleming introduced penicillin. The discovery of penicillin has been recognized as one of the greatest advances in human medicine [1]. The therapeutic potential of penicillin was first explored in the United Kingdom, and then during the World War II, the United States became the leader of antibiotic production. Even though Sir Alexander Fleming warned the world that an irresponsible use of antibiotics could lead to development of antibiotic resistance, the wide consumption of penicillin seemed to be inevitable [2]. The first bacterial resistance against penicillin was observed in the 1960s, which forced scientists to look for a remedy of this issue. The problem was solved by introducing and by developing of new series of antibiotics. In the dawn of antibiotics development, the pattern of discovering new antibiotics as a response of emerging resistance was continued, without taking into account of combating antibiotic resistance [3]. Eventually, the development of new antibiotics becomes challenging and antibiotic resistant bacteria become prevalent. These phenomena arose the necessity of rational use of the antibiotics and finding a way to stop the emerging of antibiotic resistance in the environment.

Antimicrobial resistance (AMR) is now considered as an urgent global health threat. The severity of the issue was highlighted by the World Health Organization report that testifies the increasing incidence of resistance-induced health problems in every region of the world [4]. This led to the proposition of a “post-antibiotic” era whereby common infections can no longer be effectively treated by antibiotics [5].

1.2 Environmental Aspect of Antibiotic Resistance Genes (ARGs)

Antibiotic resistance constitutes a global public health threat. It has been suggested that the contemporary resistance genes even originated in the natural environment before the first antibiotic was discovered by human [6]. Out of 30 lyophilized *E. coli* strains isolated in 1946, four were resistant to 8 different antibiotics and their resistance genes could be transferred to other *E. coli* through conjugation [7]. In another study, genes coding resistance to tetracycline, glycopeptide, and *β*-lactam were discovered in a 30,000-year-old permafrost sediment, indicating resistance exists way before mass production of antibiotics [8].

Studies have been shown that antibiotic resistant bacteria and associated resistance genes are ubiquitous in the different environment compartments – from soil and municipal wastewater systems to aquaculture facilities and animal husbandry facilities [9]. Durso et al. quantified the numbers and kinds of antibiotic resistance genes in natural and agricultural samples from 26 different environments. They reported that ARGs occur in natural, agricultural, and human-impacted habitats [10,11]. More importantly, the presence of resistance genes in different bacterial hosts suggests that resistance genes can be easily transferred between different species. Accordingly, such studies frequently state that the environment, an important reservoir of antibiotic resistant bacteria, poses a risk for human health because these genes can be transferred to bacterial pathogens [12–15]. Most of the antibiotic resistance found in pathogens is acquired via mobile genetic elements, and those resistance genes have been detected in natural environments. For example, the *qnrA* gene that is associated with fluorquinolone resistance has been

observed in aquatic environment [16].

In summary, our knowledge about abundance and occurrence of antibiotic resistance genes in environments are expanding; however, little is known about how environmental conditions may influence the rate and the dissemination of ARGs in microbial population, which hampers our ability to generate reliable comparisons and evaluation of the growth of ARG reservoirs in the environment [15].

1.3 Resistance Dissemination Mechanisms

Resistance can acquire through two distinct paths: vertical evolution, in which selection of naturally occurring resistant mutants causing the development of antibiotic resistance; or horizontal evolution, whereby acquisition of resistance genes occurs through conjugation, transformation, and transduction [17].

1.3.1 Horizontal Gene Transfer

Horizontal gene transfer (HGT) refers to the ability of microbial species to exchange genetic materials, other than genes transferring from parent cells to daughter cells [18]. Three main mechanisms of HGT are conjugation (i.e., the transfer of genetic materials between two adjacently located bacteria by plasmids), transformation (i.e., the uptake of extracellular DNA by competent bacteria), and transduction (i.e., the movement of genetic material between species by bacteriophages) [19]. Although these mechanism often happen between closely related species, they can also occur between phylogenetically distant organisms, increasing genetic variability over evolution history. It has been reported that up to 17% of *E. coli* genome and up to 25% of genomes of other

bacterial species is due to HGT event [20]. In addition, horizontally transferred genes bring about diverse functions into recipient cells, including antibiotic resistance, virulence factors, and metabolic traits [21]. Some ARGs occur on mobile genetic elements, which are members of the communal gene pool available to a wide variety of bacterial species. Therefore, the cross phylogenetic boundaries characteristic of HGT and the presence of diverse mobile genetic elements carrying ARGs make HGT the main cause of spreading antibiotic resistance in the environment [22].

1.3.1.1 Conjugation

Conjugation refers to the transferring of DNA from donor to recipient through direct cell-to-cell contact. During conjugation, one bacterium serves as the donor and another serves as the recipient. Once the two cells are in contact, either a circular double-stranded DNA (e.g., plasmid) or a single stranded DNA will be transferred from the donor to the recipient [23]. The new genetic materials often provides some genetic advantage to the recipient [24]. For example, the majority of genes transferred from donor to recipient are ARGs which help recipient cells survive the presence of antibiotics. These genes are typically transferred resistance in the form of plasmids [25].

A plasmid is an extrachromosomal genetic element that can independently replicate. They are usually much smaller than the bacterial chromosome, varying from less than 5 kbp to more than several hundred kbp, though plasmids as large as 2 Mbp (i.e., megaplasmid) occur in some bacteria [26].

Plasmids that can self replicate and that carry the genes needed for self-transmission are called conjugative plasmids. Plasmids that can only be transferred by an outside machinery are called mobilizable plasmids [27]. Plasmids are found in a wide variety of environments and can be transferred across taxa. In addition, most broad-host-range conjugative plasmids have been found carry ARGs [28]. Conjugation is often regarded as the main mechanism for the horizontal transfer of ARGs [29].

1.3.1.2 Transformation

Transformation is a process of HGT in which a bacterium acquires exogenous DNA from the environment. Natural competence or the ability to acquire DNA from the environment is a complex process [30]. Transformation requires the expression of specific genes and demands specific conditions. Some researchers argue that transformation does not play a major role in spreading antibiotic resistance genes [31].

1.3.1.3 Transduction

Transduction refers to a process in which bacteriophages transfer DNA from one bacterium to another. Transduction occurs when the phage attaches to its cognate receptor on a host. Then, the phage integrate its genome into host genome and synthesize the new genome to replicate [32]. Due to the specificity between a phage and its host, it is believed that transduction also has less impact on the dissemination of antibiotic resistance genes between bacterial genera. However, further investigation is warranted as transduction does not require physical contact (which is required in conjugation) and is DNase resistant (as opposed to transformation).

1.3.2 Vertical Gene Transfer

In 1859, Charles Darwin introduced term mutation whereby population gradually evolves over the course of generations through natural selection [33]. Mutation happens as a result of errors in DNA replication during the transmission of DNA from parents to offspring. Mutation is regarded as an most important process in bacterial evolution as it can happen in every generation [34]. Mutation is typically defined as heritable changes in genotype that can occur spontaneously or be induced by a pressure in the environment such as chemical presence, such as the emergence of antibiotic resistance in the presence of antibiotics [26]. Most of the mutations are nonfunctional and, thus, exchanging genetic materials between different species seems more concerning in spreading of antibiotic resistance [35]. The key parameter in mutation is time and generation time. The higher the number of generation there is, more mutations can potentially happen [36].

1.4 Antibiotic Resistance in Surface Water/Wastewater Treatment Plant

Wastewater treatment plants are considered as an important reservoirs of antibiotic resistance genes and hotspots for HGT, due to high diversity of bacteria, high nutrient availability, and potential of biofilm formation [37–40]. In addition to high diversity and abundance of organisms, sewage has the trace of heavy metals, residuals of drugs, pesticides, and many other toxic chemicals [41–43]. Even advanced wastewater treatment technologies cannot completely remove those contaminants [44]. For example, the removal efficiency of tetracycline in wastewater treatment has been reported from 12% [45] to 80% [46]. Hence, the treated effluent of WWTPs is still comprised of pollutants that not only can

be entered to surface water and soils, and ultimately the chain food, but also can exert a selective pressure, which may facilitate HGT. Moreover, biofilm formation (i.e., sludge) as well as interface of different environment matrices may accelerate genetic exchange by conjugation [47]. A wide variety of broad-host-range plasmids, which can independently transfer and replicate in broad range of phylogenetically distinct hosts, are often found in WWTPs [48]. Besides, broad-host-range plasmids can transfer mobilizable transposons (nonself-transferable plasmids) encoding antibiotic resistance genes to hosts. WWTP thus is a reservoir of communal antibiotic resistance genes and conjugative plasmids [25,49–51].

Some studies argue that preexisting genetic diversity and environmental selective pressure in WWTPs can lead to horizontal and vertical gene transfer [52–56]. However, those studies used either unrealistically high concentration of antibiotics (mg/L) or optimized conditions in terms of stress for bacteria population, which is not typical of the environment. Indeed, the level of antibiotics that have been detected in surface water, sewage, hospital effluents, and groundwater is generally on the orders of magnitude of μg/liter and even ng/liter [57–59]. Moreover, the fastest rate of plasmid transferring has been observed at antibiotic concentration at around 10 μg/L [60]. In the surface water environments, bacterial populations are also influenced by environmental conditions. However, the effects of environmental conditions on the conjugation frequency has not been systematically studied.

1.5 Ecological/Environmental Factors

Even though the role of conjugation in disseminating ARGs has been recognized, how environmental conditions affect conjugations under real environmental conditions is

not well understood. Generally, conjugation takes place when donor/recipient overcome physiological, mechanistic, and selective barriers in the process [19,61]. This means the physiological state of donor and recipient as well as energy availability might drive the process before and/or during conjugation. In a recent study, Hiltunen et al. used the eco-evolutionary dynamics framework to emphasize on community in spreading ARGs (Figure 1.1). They highlighted that eco-evolutionary feedback is one of the reasons of rapid evolution that we counter today. They argued that how combination of variety of environmental factors – such as nutrient availability and antibiotic at sub-MIC level – can change the population density and dynamics, and subsequently these changes cause evolution. When evolution emerges, it can change the function(s) and/or structure of the population [62]. Therefore, in order to better understand conjugation in different environmental compartments, a closer look at environmental/ecological factors that affect population size and dynamic is appropriate.

Some of important environmental parameters that can affect conjugation rate include: nutrient availability, temperature, pH, variation between different species, and the environmental matrices [63,64]. Although these environmental parameters are measureable and quantifiable with current technologies, investigating the effect of individual environmental parameter on conjugation frequency has been challenging. One main reason is that gene transfer, particularly plasmid transfer, has been known as an extremely complex process as it requires of simultaneous occurrence of several factors to complete [65]. In this regard, Lopatkin et al. investigated the effect of an antibiotic on conjugation rate and demonstrated that the presence of an antibiotic does not necessarily promote conjugation efficiency[66]. This statement is contradictory to traditional belief that

antibiotics can increase conjugation rate due to its selective pressure impact [67]. Antibiotics can indirectly affect a wide variety of ecological parameters such as population sizes, bacterial community composition, defense mechanisms in populations due to cost of antibiotic resistance, and growth rate of bacteria. For example, if an antibiotic does not affect both donor and recipient, there would not be any advantage for transconjugants. Therefore, these results suggest that in a real-world scenario the interaction and/or combination of different factors on different bacterial community define conjugation mechanism.

Our understanding about conjugation efficiency can be advanced by considering the effect of different environmental parameters separately. For example, it has been suggested that the abundance and growth rates of different microbial populations play a crucial role in conjugation rate [66]. Growth rate is affected by multiple environmental factors such as pH and temperature. In conclusion, the nutrient levels – which is related to cell density – and growth rates of bacteria seem to determine the extent of conjugation under various environmental conditions [64,68].

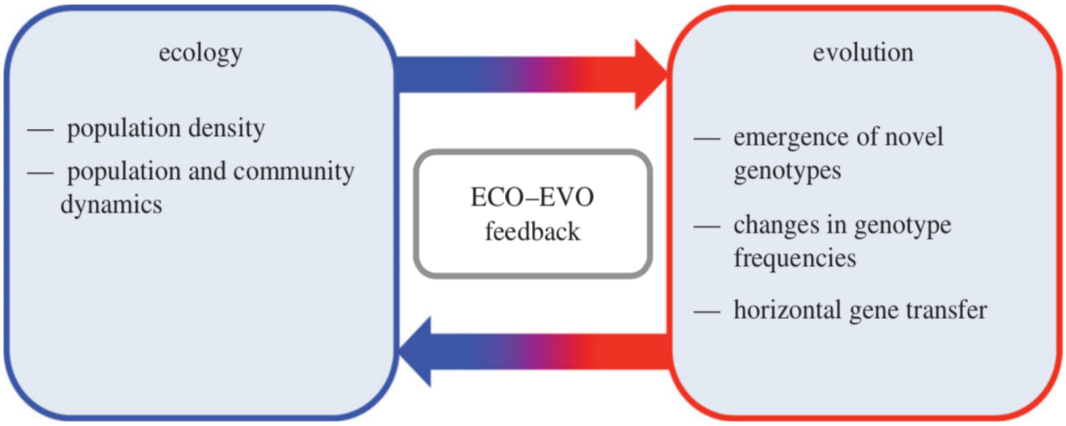


Figure 1.1. Schematic of eco-evolutionary feedback loop [62].

1.6 Objectives

As ARG-carrying bacteria enter receiving water through WWTP discharge, they interact with indigenous microbes under various environmental conditions. Background nutrients and growth rates represent important environmental factors that can affect conjugation process. The following objectives were investigated in this thesis:

I. Understand the effects of bacterial growth rates and background nutrient levels on ARG conjugation frequency and mutation rate in a simulated river setting

II. Compare how the resistance level of recipient changes due to conjugation and mutation under different environmental conditions

III. Characterize the plasmids and mutations observed in part I and II.

1.7 Overview of Approach

Batch reactor or filter mating approaches [69] cannot decouple the effects of multiple parameters on conjugation. Hence, chemostat reactors are proposed as they allow for separate investigations of growth rate and bacterial density [70]. Continuous cultures like chemostat can better simulate the natural environment such as river comparing to batch reactors. Two important environmental factors (i.e., nutrient availability and growth rate) were tested for their effects on horizontal and vertical transfer of tetracycline resistance genes [71]. Tetracycline was chosen as it is a widely used antibiotic and is one of the most detected antibiotic in the surface water environments [72,73]. Environmentally relevant tetracycline concentration (i.e., 10 μg/L) was introduced to the system with donor to mimic the discharge of WWTPs to the environment [74,75]. In order to elucidate the molecular mechanisms underlying the changes in MIC following horizontal or vertical gene transfer, whole genome sequencing (WGS) was adopted. The insight from bioinformatics/sequencing can reveal the molecular mechanism behind phenotypical changes in bacteria.

1.8 Thesis Outline

This thesis consists of five chapters. Chapter One is a literary overview of antibiotic resistance history, environmental framework of ARGs, antibiotic resistance spreading mechanisms, and ARGs in surface waters. Chapter Two describes the protocols and methodology used in this thesis. Results and discussion are presented in Chapter Three. In Chapter Four, conclusions are summarized and ideas for future work are

suggested. References and appendices including experimental setups, suppliers and parts and supplementary information are listed at the end of this thesis.

CHAPTER 2. MATERIALS AND METHODS

2.1 Strains and Chemicals

Two *Escherichia coli* strains CV601 and J53 were obtained from Dr. Holger Heuer of Julius Kühn Institute as donor and recipient cells, respectively, for the conjugation study. This pair of strains have been used as model bacteria to study ARG proliferation in several other studies [76–81]. *E. coli* strain CV601 gfp is resistant to both kanamycin and rifampicin and is tagged with gfp, which makes the cells green under UV light [82]. The *E. coli* strain J53 (F^- met pro Azi^r), which is resistant to sodium azide, was used as recipient cells in the conjugation experiments [83].

Tetracycline antibiotics often occur in wastewater streams entering the aquatic environment, and their corresponding ARGs in rivers receiving the wastewater streams [84–86]. Tetracycline was used as the model antibiotic in this study. Tetracycline hydrochloride (Sigma Aldrich, St. Louis, MO) stock solution, at 512 mg/L, was freshly prepared and stored for no more than 7 days in 4°C. The stability of the stock solution over the storage period was checked using high-pressure liquid chromatograph (HPLC) in the Water Sciences Laboratory at UNL.

2.2 Acquisition of Plasmid(s) Containing ARGs

Filter mating was employed to capture conjugative plasmids in the treated effluent of Lincoln Theresa Street WWTP using *E. coli* CV601 as recipient cells. The filter mating procedure used in this study was as described by Heuer et al. with slight modifications including time of incubation, adding kanamycin to the media, and temperature of incubation [82]. Figure 2.1 shows the flowchart of the procedure.

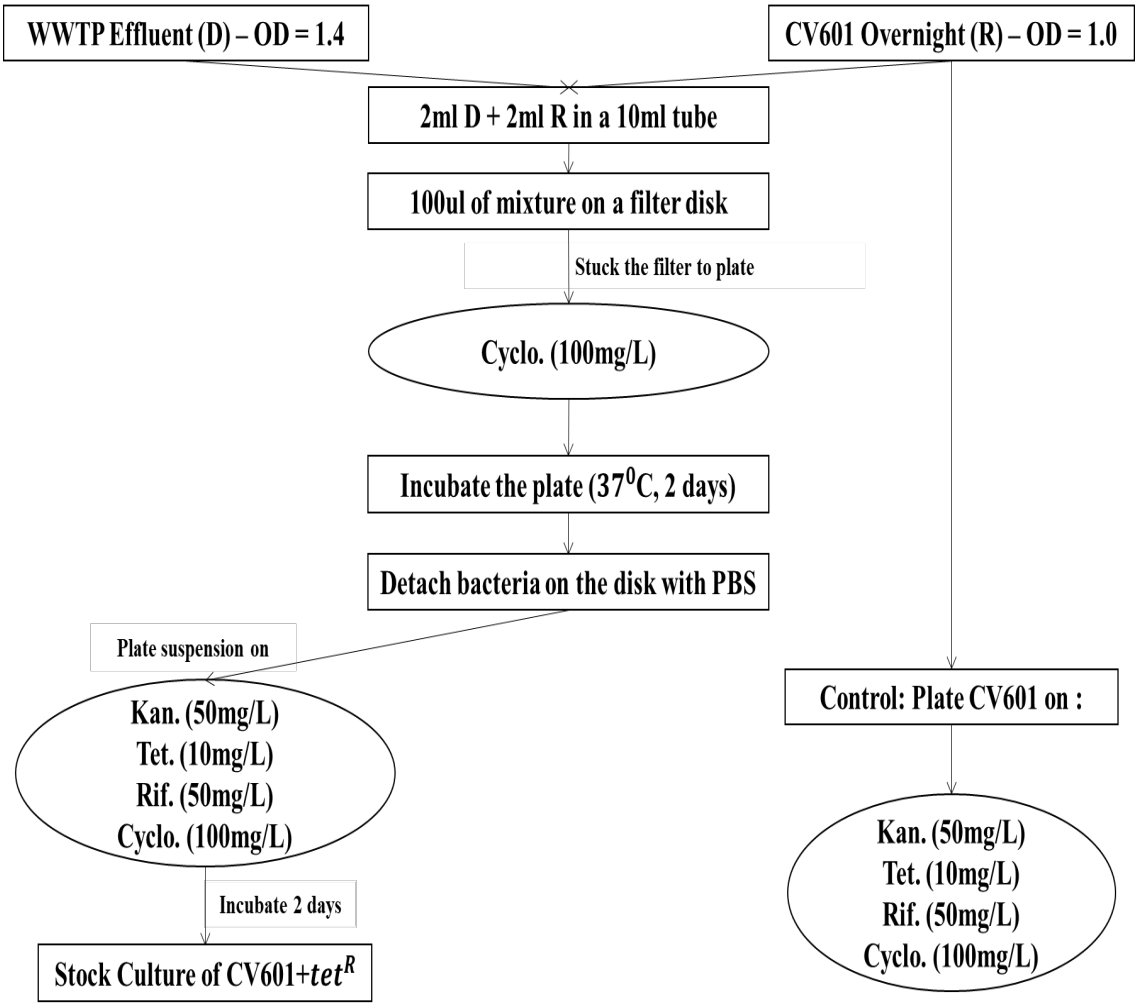


Figure 2.1. Flowchart for capturing of conjugative plasmids containing tetracycline resistance genes from the treated effluent of Lincoln Theresa Street WWTP.

The glycerol stock of *E. coli* CV601 was cultured in lysogeny broth (LB) in the presence of kanamycin (50 mg/L) and rifampicin (50 mg/L) with shaking at 30°C overnight. The cell culture was then diluted 20 folds in fresh LB broth and grown for 10 hr to have recipient community in their exponential growth phase (Figure B1). The cells were washed twice in phosphate-buffered saline (PBS) to remove the trace of antibiotics and re-suspended in 15 ml of PBS.

Treated effluent was filtered through 0.45 μm pore size S-Pak filters (Millipore Corporation, Bedford, USA). Bacteria were removed from the filters by vortex for 15 min in 25 mL PBS. The bacterial suspension was decanted to a new tube and centrifuged for 15 min at 2700 ×g. rpm. The pellet was washed with PBS twice and re-suspended in PBS. The volume of PBS was adjusted to reach an optical density at 600 nanometers (OD_{600}) of 1.4.

2 ml of effluent bacteria and 2 ml of CV601 were mixed and vortexed. 100 μL of mixture was loaded onto a 0.22μm pore size mixed cellulose ester filter (Whatman plc inc., Maidstone, Kent, UK). Then, the filter was placed a LB agar plate amended with 100 mg/L cycloheximide and incubated at 37 °C for 2 days. Cycloheximide is an active antibiotic against many molds, yeast, and phytopathogenic fungi [87]. Therefore, we used cycloheximide to remove undesirable organisms from WWTPs sample. After incubation, the cell mixture on the filter disk was re-suspended in 2 mL PBS through vortex. The suspension was plated on LB agar plates, which contained kanamycin (50 mg/L), rifampicin (50 mg/L), cycloheximide (100 mg/L), and tetracycline (10 mg/L). As controls, CV601 donor cells were grown and plated under the same conditions except the

presence of cells from treated effluent. After 2 days, CV601 transconjugants receiving plasmids from the treated effluent formed visible colonies on agar plates and their identity was further verified using green fluorescence under UV light. The confirmation of capturing *tet*-carrying plasmids was rechecked by minimum inhibitory concentration test. The resulting CV601 (designated as "CW"), which contained *tet*-carrying plasmids, was inoculated in selective liquid media and prepared as stock culture in glycerol and stored in -80 °C freezer.

2.3 Chemostat Reactor Design

The experiment was conducted according to a 2×2 factorial design: growth medium (1/3-strength and 1/10-strength Mueller Hinton Broth (MHB)) and growth rate (0.15 and 0.45 hr^{-1}). Two *E. coli* strains, CV601 transconjugants and J53, were used as donor and recipient, respectively. First, *E. coli* J53 were cultured in LB broth in presence of sodium azide (200 mg/L) at 35^{o} C shaking overnight. Then, it was diluted to adjust OD of 0.3, i.e., exponential phase (Figure B2). Finally, fresh *E.coli* J53 used in chemostat to establish a steady state before CV601CW cells were introduced. All reactors contained 15 mL of *E. coli* J53 to simulate recipient cells in surface water. The experimental setup was largely adopted from Dunham et al. with slight modification [71,88].

In each run, three replicate chemostat reactors were operated for one treatment combination. The chemostat systems were established according to the procedure of Dunham lab ministat manual [71]. Parts and part numbers needed to establish the chemostat reactor system is provided in Table A1. In brief, each chemostat reactor is made of a 50 mL pyrex tube with a 4-hole cap. The reaction solution volume is 30 mL. The growth

medium was delivered from a 5 L carboy via a Watson Marlo pump at either 4 or 10 mL/hr to obtain the desired specific growth rates. Within each experimental run, all reactors had the same dilution rate (i.e., 0.15 or 0.45 hr^{-1}). In chemostat reactors, the growth rate of a single culture is equal to the dilution ratio of the reactor at steady state. To monitor if steady state has been reached, the cell density of recipient cells was monitored by measuring optical density When the density of recipient cells reached steady state in the reactors, tetracycline resistant CV601 donor cells and tetracycline were added to the chemostat reactors through another carboy to reach final concentrations of 10^8 CFU/mL and 10 μg/L in the chemostat reactor. The time that the donor and antibiotic was added was denoted as Day 0. 15 mL donor directly add to reactors and then the donor line was inserted to the pump with the same flowrate as media. Since the flow rate into reactor became double and the reactor volume also went up from 15 to 30 mL, the dilution ratio kept constant. The experiments were repeated at least once for each dilution ratio (i.e., 0.45 and 0.15 hr^{-1}).

To account for the emergence of ARGs due to mutation, all the chemostat experiments were repeated in the same manner without introducing the donor.

The samples were taken after 16, 24, 48, 72, and 96 hr to monitor conjugation events. The number of donor, recipients and transconjugants were quantified using culture-based method. At the end of experiments, samples were taken for whole-genome sequencing.

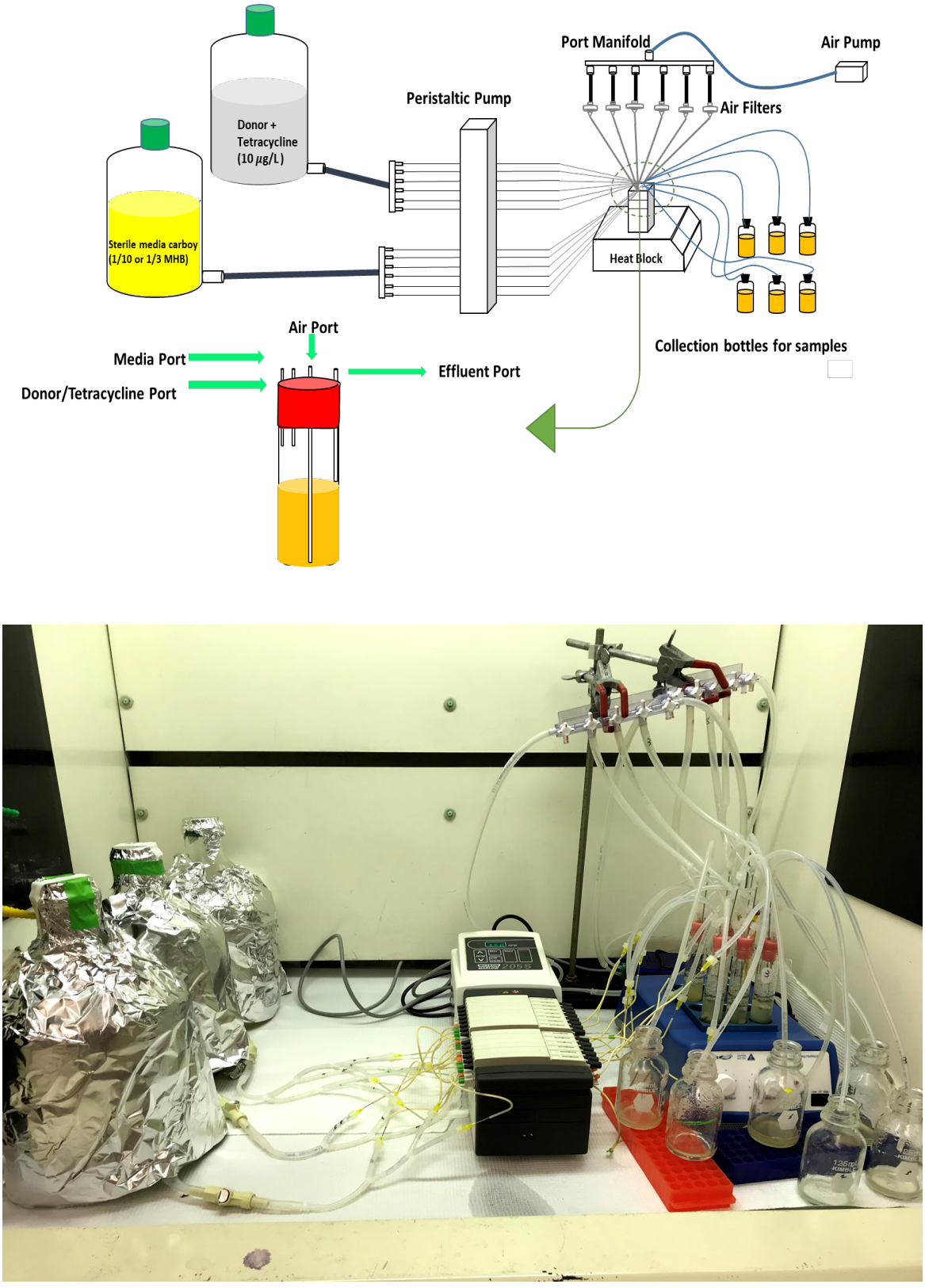


Figure 2.2. Schematic arrangement of chemostat reactor.

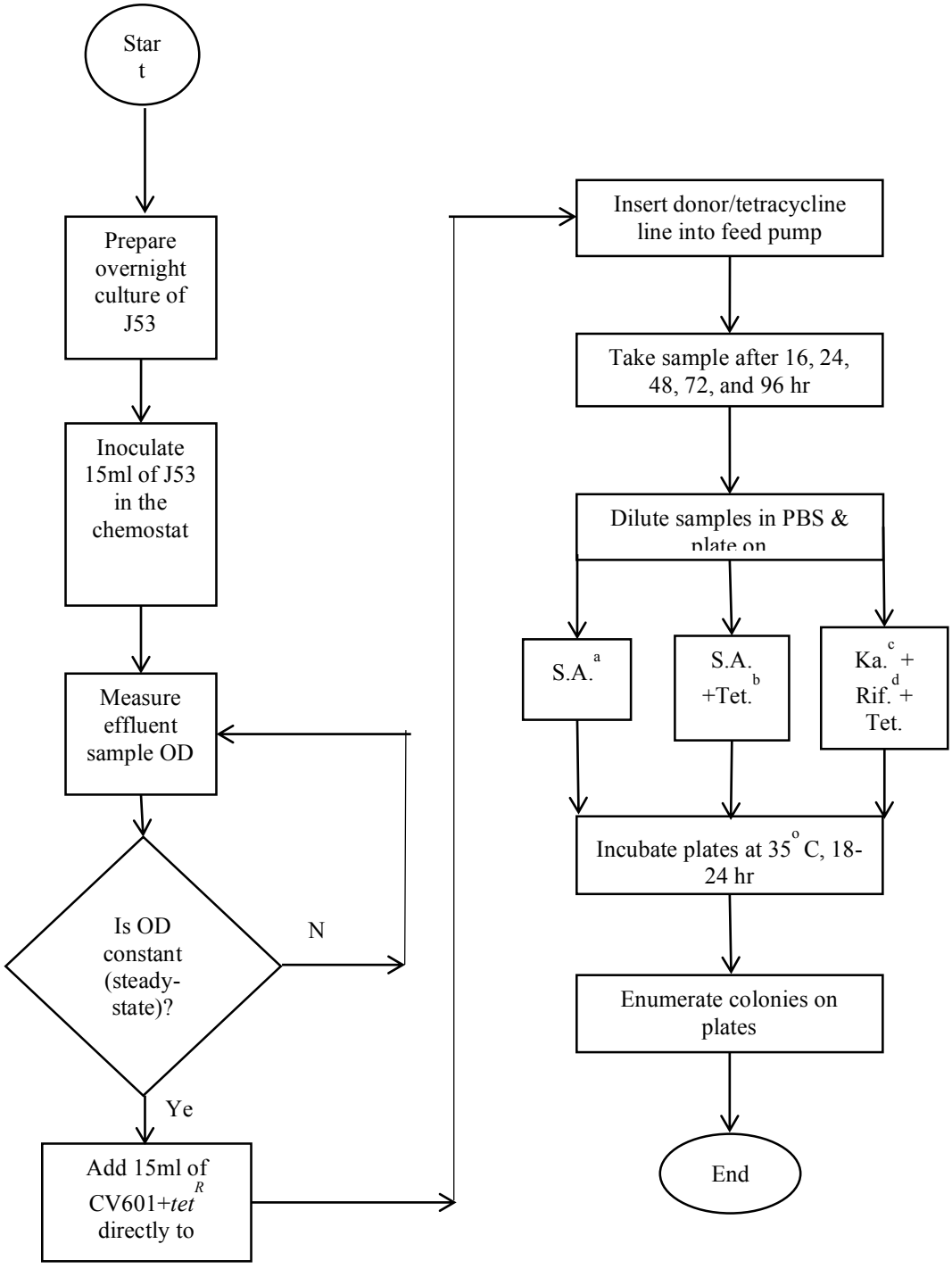
2.4 Bacterial Enumeration

The viable plate count method was used to monitor number of transconjugants, recipient, and donor in order to calculate conjugation frequency and control the experiment conditions. All the samples were diluted in a 10-fold dilution series for each reactor/technical replicate. For each dilution, plating was performed in triplicates. Colony forming units (CFUs) for each run and plate was determined using the mean of the plate triplicates, followed by the mean of the technical replicates (three per run).

For donor, frequent sampling events from the feed line and the rectors containing donor cells was serially diluted in PBS and was plated on selective LB plates. After incubation of 16 hr in 35^{o} C, donor were selected on LB plates supplemented with kanamycin (50 mg/L), rifampicin (50 mg/L), and tetracycline (10 mg/L).

Recipient cells were frequently sampled from the effluent line. Mixed culture were serially diluted in PBS. Then, diluted recipient cultures were plated on LB plates with sodium azide (200 mg/L). After 20 hr in 35^{o} C, the number of recipient was recorded.

For the selection of transconjugants, cultures directly were sampled from the effluent of chemostat reactors and after serially diluted in PBS, they were plated on LB supplemented with tetracycline (10 mg/L) and sodium azide (200 mg/L). The plates were incubated for 24 hrs at 35^{o} C. The same procedure was used to check mutation events in recipients from mutation experiment.



[a]Sodium azide.
[b]Tetracycline.
[c]Kanamycin.
[d]Rifampicin.

Figure 2.3. Workflow of differentiate of donor, recipient, and transconjugants in chemostat reactor experiment.

2.5 Calculating Conjugative Frequency

Conjugation frequency was expressed as the number of transconjugants per recipient colonies formed as in equation 1 [89].

Equation 1: $$Conjugation\ Frequency = \frac{\#\ of\ Transconjugants(\frac{CFU}{mL})}{\#\ of\ Recipients\ (\frac{CFU}{mL})}$$

2.6 Minimum Inhibitory Concentration Measurement

For the broth microdilution, stock tetracycline solution was serially diluted using MH media into two-fold concentrations corresponding to 256 to 0.5 mg/L and carefully dispensed into 96-well plate (VWR Company, Pennsylvania, USA). Inoculums of the donor, recipient or transconjugants was diluted and the inoculated in each well except the negative controls of the 96-well plate to achieve approximately 110 µL final volume in each well. The plates were then incubated in a microplate reader (Biotek instruments, Inc., Vermont, USA) at 37 ºC for a period of 16-20 hours to obtain growth curves by continuous absorbance reading during incubation period. After the incubation period, the plates were examined to further identify the MIC. The MIC was taken at the lowest concentration of tetracycline that did not permit any visible growth [90].

Even though MIC is a single time-point measurement made after a fixed incubation time, with the reader, it was clear that there is a substantial drop in light scattering at the MIC compared to lower inhibitor concentrations.

2.7 Whole Genome Sequencing and Assembly

Seven samples from different circumstances were chosen and sequenced using Illumina Hiseq technology (Table 2.1). Total DNA, including plasmids and chromosomes, was extracted using Qiagen DNA kit (Qiagen, Inc, Germantown, MD). Sequencing reads, gene and plasmid assembly, and genome annotation analysis were conducted in the Center for Biotechnology at University of Nebraska-Lincoln.

First, sequencing quality control was performed by FASTQC to check GC content and quality of reads. Then, TrimGAlour was used to trim low quality bases from the 5-prime and 3-prime reads and to remove reads that have on average low quality (i.e., Q30 and lower). Reads that contained no base were also removed. The remaining reads were used to generate assembly via SPAdes (bacteria assembler) with multiple Kmer values. After assemblies, the quality of each assembly was analyzed using QUAST. The average sequence read length was 368 nucleotides and a total of 255 Mb was sequenced. Genome annotation was conducted using Prokka with settings: kingdom bacteria and genomic code 11. We allowed for overlaps between the coding regions of genes and rRNAs region. In addition, we looked for noncoding RNAs in annotation. KAIJU looked at raw reads and determined the lineage and family of bacteria. We uploaded the full genome to Plasmid Finder to identify potential plasmids. We also utilized PLSDB database to confirm the presence of the plasmids. Bowtie 2 was used to align samples. SAMtools and BCFtools were utilized to generate and filter variant coding format files.

Table 2.1 Samples for genomic sequencing.

Sample #	Culture	Dilution rate	Nutrient concentration	Exp. Condition
1	Transconjugant	0.15 hr^{-1}	1/3 MHB	Conjugation
2	Transconjugant	0.45 hr^{-1}	1/3 MHB	Conjugation
3	Recipient	0.15 hr^{-1}	1/3 MHB	Mutation
4	Recipient	0.45 hr^{-1}	1/3 MHB	Mutation
5	J53	-	-	Original Culture
6	CV601	-	-	Original Culture
7	CV601+Tet	-	-	Transconjugant from WWTP

2.8 Statistical Analysis

Multivariate analysis of variance (MANOVA) was conducted to evaluate the significance of two nutrient backgrounds and growth rates on conjugation frequency and number of transconjugants for each time point. All the statistical analysis was carried out using TIBCO Statistica™.

CHAPTER 3. RESULTS AND DISCUSSION

3.1 Establishing Steady State in Chemostat

One of the most important features of chemostats is that microorganisms can be grown in a physiological steady state under constant environmental conditions [91]. In this steady state, growth occurs at a constant specific growth rate and all culture parameters remain constant (i.e., culture volume, nutrient and product concentrations, pH, cell density, etc.) [92]. Therefore, one can independently set and control growth rate, cell density, and selection pressure. In a chemostat reactor, dilution rate is the rate of media volume addition relative to the reactor volume. At steady-state, the growth rate of bacteria is equivalent to the dilution rate applied to chemostat reactors (Brock, 2012; Herbert, Elsworth and Telling, 2009; Ziv, N., Brandt, N. J., Gresham, 2013). This enables one to control growth rate of the culture by simply changing the speed of the feeding pump.

The recipient cell J53 was inoculated in reactors and the speed of the pump was adjusted to result in desirable dilution ratios (i.e., 0.45 or 0.15 hr^{-1}). The literature reports that the growth rate of *E. coli* in the environment has a range of 0.17 to 0.9 hr^{-1} [95]. Considering the washout rate and the bacteriostatic effect of tetracycline, the dilution rates of 0.15 hr^{-1} and 0.45 hr^{-1} were chosen to represent the low and the high growth rate, respectively, in this study. The cell density in the reactor was monitored to determine if steady state has reached. Reactors reached steady state after 1.5 and 3.5 days under high and low dilution ratio, respectively (Figure 3.1). After reaching steady state, the growth rate of recipient was technically set on dilution ratio and donor exposed to the reactors for conjugation experiment.

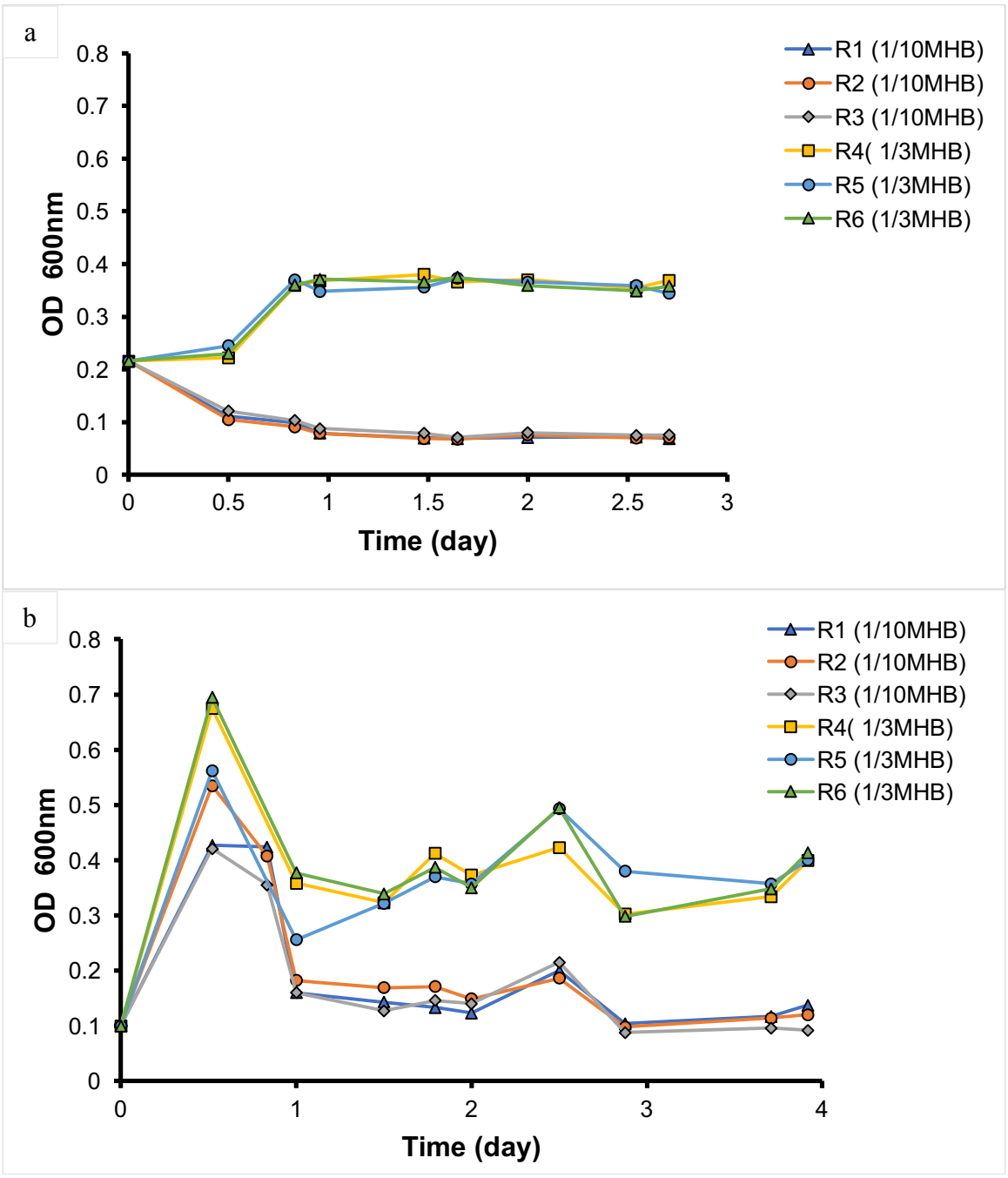


Figure 3.1. Density of recipient cells in triplicate chemostat reactors for 1/10 MHB and 1/3 MHB under 0.45hr^{-1} (a) and 0.15 hr^{-1} (b) dilution ratio.

3.2 The Effect of Growth Rate on Conjugation Frequency

The influence of growth rate on conjugation frequency was investigated.

Tetracycline was added to the chemostat reactors at an environmentally relevant

concentration (i.e., 10 µg/L) to provide selective pressure [58,59]. In addition, this concentration appears to be sufficient to provide selective pressure for the transfer of ARGs [60].

The higher growth rate led to higher conjugation frequencies (Figure 3.2). Under the nutrient level of 1/10 MHB, the conjugation frequency was $8.08 \pm 4.41 \times 10^{-4}$ and $1.81 \pm 2.21 \times 10^{-6}$ at 16 hr for high and low growth rate, respectively, following the addition of donor cells and tetracycline to recipient cells in the chemostat reactor. In comparison, at the nutrient level of 1/3 MHB, the conjugation frequency was $8.94 \pm 1.76 \times 10^{-5}$ for bacteria grown under 0.45 hr^{-1} and $1.87 \pm 2.48 \times 10^{-6}$ under the growth rate of 0.15 hr^{-1}. The results were reported only for 16 hr mating duration.

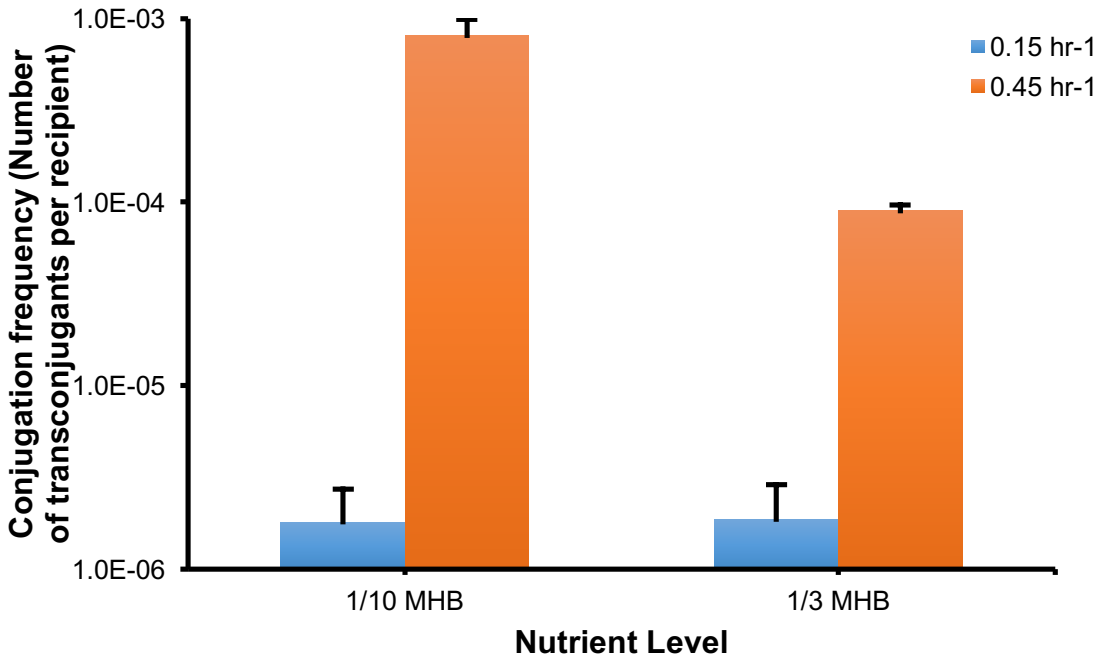


Figure 3.2. The effect of growth rate on conjugation frequency for 1/10 MHB and 1/3 MHB at 0.45 hr^{-1} and 0.15 hr^{-1} growth rate, in 16 hr mating duration.

In the scientific literature, traditional conjugation procedures use a mating time window of 16 hr. After 16 hr, the subsequent growth of transconjugants may overestimate the conjugation frequency. Due to this fact, conjugation frequency is only applicable in the traditional time window (i.e. 16 hr) [96]. However, bacteria can share the same habitat for days and even years in the environment, resulting in drastically increased mating times. Therefore, the number of transconjugants was compared in lieu of the conjugation frequency for the time points after 16 hr (Figure 3.3). Under the 1/10 MHB nutrient level, the number of transconjugants increased from $9.83 \pm 1.95 \times 10$ to $4.89 \pm 4.37 \times 10^3$ CFU/mL and from $5.08 \pm 5.32 \times 10$ to $3.69 \pm 3.38 \times 10^2$ CFU/mL for 0.45 hr^{-1} and 0.15 hr^{-1}, respectively (Figure 3.3. a). Similarly, under higher nutrient level (i.e., 1/3 MHB), the number of transconjugants showed an increase from $2.10 \pm 0.32 \times 10^2$ to $4.19 \pm 2.88 \times 10^4$ for cultures grown under 0.45 hr^{-1}. For bacteria grown at 0.15 hr^{-1}, the number of transconjugants was lower and in the same range for the course of experiments – $1.18 \pm 1.21 \times 10^2$ to $6.61 \pm 6.18 \times 10^2$ (Figure 3.3. b).

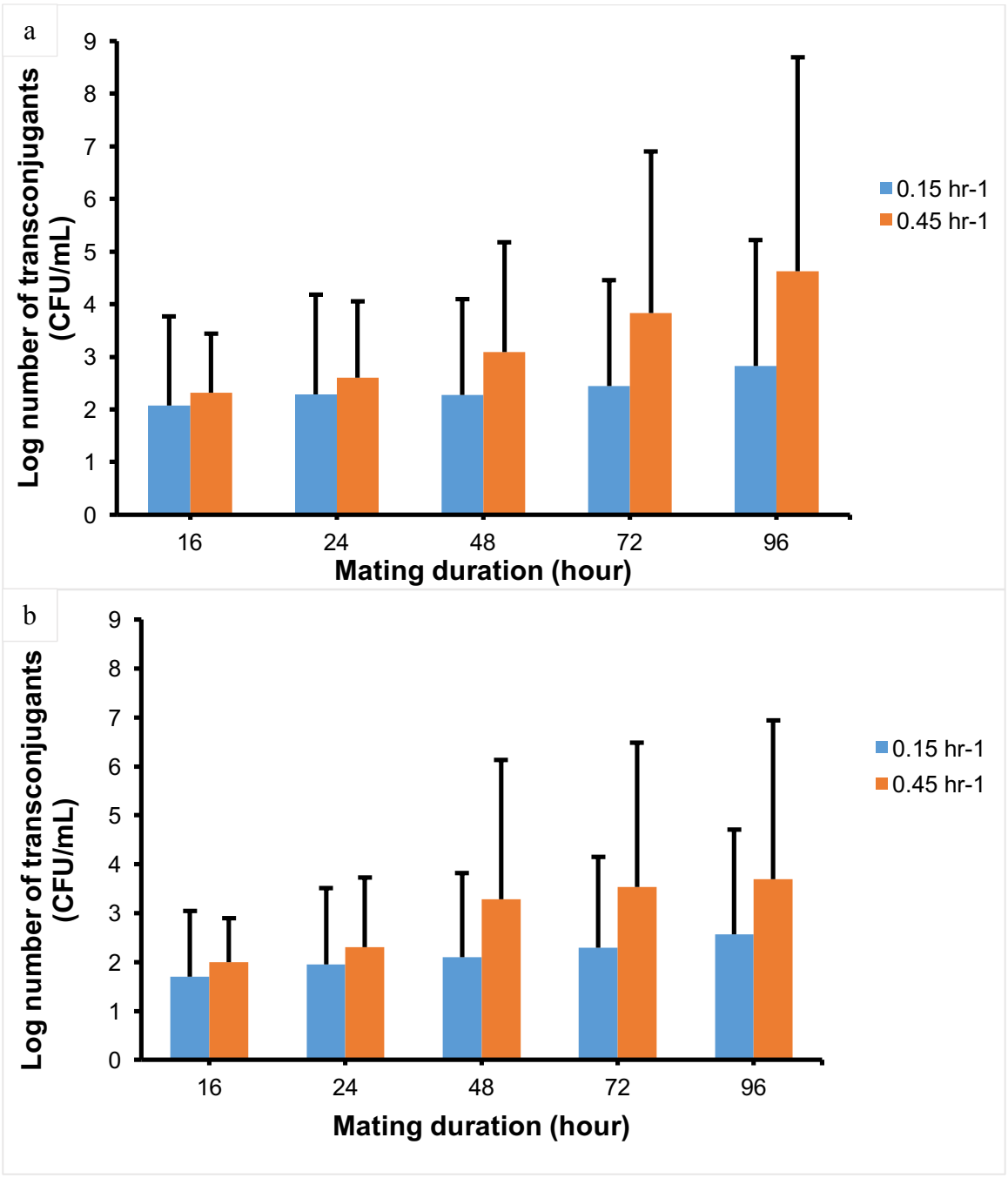


Figure 3.3. The effect of growth rate on number of transconjugants at 0.45 hr^{-1} and 0.15 hr^{-1} growth rate for a) 1/10 MHB and b) 1/3 MHB nutrient level. Errors bars are from six replicate chemostat reactors.

Main and interaction effects of nutrient level and growth rate were assessed using a multivariate repeated measures analysis of variance, where response variables were log-transformed concentrations of transconjugants and replicates. An identical repeated-measures ANOVA was also conducted where conjugation rate was the response variable. MANOVA showed significant main effects of both nutrient level ($F_{2, 19}$ = 30.5, $P < 0.001$) and growth rate ($F_{2, 19}$=246.3, $P < 0.001$), with Tukey's post-hoc test showing significantly higher in lower nutrient level ($P < 0.001$) and higher growth rate ($P < 0.001$). MANOVA also detected a significant interaction effect of nutrient level and growth rate ($F_{2, 19} = 5.1$, P=0.02), with post-hoc tests showing significantly higher at 0.45 hr^{-1} growth rate (P = 0.004) and 1/10 MHB (P = 0.0170).

These results can be explained by two concepts: 1) different number of generations for the two growth rates at each time point; 2) fitness cost to bacteria in the community.

For a given experimental duration, a higher growth rate means a larger number of generations. The doubling time associated with the growth rates of 0.45 hr^{-1} and 0.15 hr^{-1} was 1.54 hr and 4.62 hr, respectively. In other words, the number of generations of the cultured cells associated with the dilution rate of 0.45 hr^{-1} was always three times the number of generations associated with the dilution rate of 0.15 hr^{-1}. For example, after 72 hr of the introduction of donor cells and antibiotic, 48 generations had occurred under the dilution rate of 0.45 hr^{-1} while only 16 generations had occurred for 0.15 hr^{-1}. Interestingly, the number of transconjugants at the same generation numbers were the same for the similar nutrient condition (Figure C1). In fact, the trend of increased number

of transconjugants showed that cultures grown under similar nutrient concentration had the same number of transconjugants at the same generation numbers, regardless of the growth rate in the reactors. Therefore, slow growing bacteria can show the similar number of transconjugants compared to fast grow bacteria if they reach at the same generation numbers.

Another well-studied reason for these results is fitness cost. Previous studies showed that HGT can provide an initial selective advantage or may subsequently affect the fitness cost of the recipient [97–99]. Adaptive genes provide a selective advantage for the recipient and have a higher chance of persisting over long periods of time [24]. As their frequency in the population increases over time, these genes become fixed. Since the number of generations was lower at a low growth rate, the transferred genes had not yet fully adapted to the genome and regulatory environment of the recipient. Therefore, it is possible that the plasmid did not decrease the fitness cost of transconjugants with a low growth rate and therefore transconjugants were unable to take over the community population. On the other hand, new genes in the recipient may fix faster and consequently provide a selective advantage to the recipient with a high growth rate during the course of the experiment, resulting in a higher number of transconjugant. It is worth noting that a previous study showed that 10 μg/L of tetracycline increased the transfer frequency by two-fold for several ARGs [60], indicating that optimal experimental conditions contribute to the transfer of the plasmid and had the highest transfer frequency in both scenarios. Moreover, fitness cost is an important factor for a bacterium in a population, especially when a stressor is present. Thus, 10 μg/L tetracycline and competition for food sources can result in cells with lower fitness costs, which can dominate the environment over

time. Higher growth rates placed a dual stress on bacteria as compared to low growth rates. Therefore, with a higher growth rate, it is likely that the recipient (J53) was outcompeted by transconjugants.

As previously mentioned, donors can be either CV601 or transconjugants J53 after the initial conjugation event. Since conjugation is a cell-to-cell contact process, increasing the number of potential donors increases the chance of plasmid transfer. However, the effect of secondary transconjugant donors was ignored because we were unable to confirm that J53 was as capable as the original donor for transferring plasmid.

3.3 Effect of Nutrient Background on Conjugation Frequency

Nutrient concentration is one of the most impactful parameters affecting conjugation efficiency [100]. The effect of nutrient level on conjugation frequency was investigated at 16 hr time-point (Figure 3.4). Under the growth rate of 0.45 hr^{-1}, the conjugation frequency was $8.94 \pm 1.76 \times 10^{-5}$ and $8.08 \pm 4.41 \times 10^{-4}$ for high and low nutrient level, respectively. In comparison, at the growth rate of 0.15 hr^{-1}, the conjugation frequency was $1.87 \pm 2.48 \times 10^{-6}$ for bacteria grown under 1/3 MHB and $1.81 \pm 2.21 \times 10^{-6}$ under the grow of lower nutrient level (i.e., 1/10 MHB).

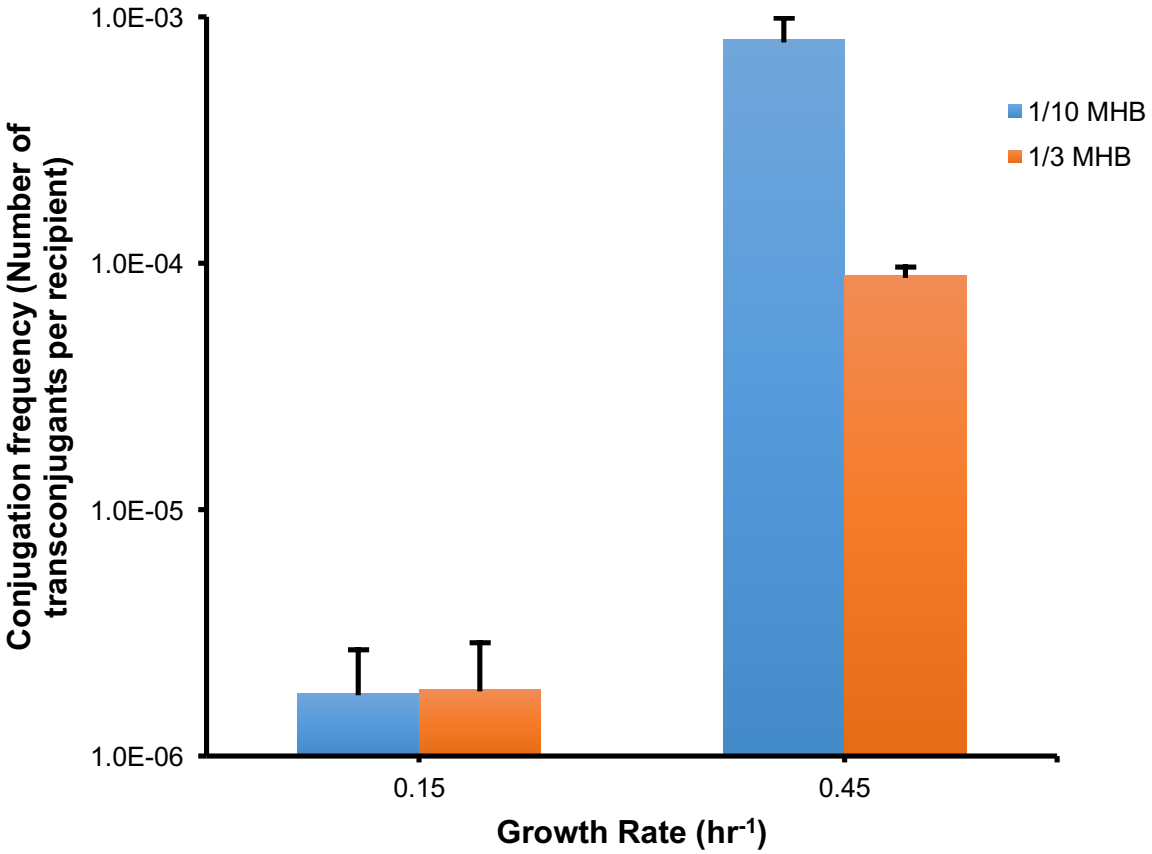


Figure 3.4. The effect of nutrient level on conjugation frequency at 0.45 hr^{-1} and 0.15hr^{-1} growth rate for 1/10 MHB and 1/3 MHB nutrient level, in 16 hr mating duration.

Although a lower nutrient background was associated with a slightly higher transfer frequency at 0.45 hr^{-1}, ANOVA showed that there was no significant effect of nutrient level on conjugation rate, however a main effect was observed for growth rate ($F = 90.3$, $P < 0.001$); thus, nutrient concentration had no effect on transfer frequency. This result is unexpected, as Lopatkin et al. (2016) showed that conjugation frequency significantly increased when the concentration of glucose was increased in the medium. However, one reason for this contradictory result is Lopatkin et al. conducted their experiment in a batch system and in the absence of additional stressors, indicating that

competition between donors, transconjugants, and recipients was not considered in their experiment. Since the energy source (i.e., nutrient level) is correlated with growth rate in a batch system, increased energy availability leads to a faster growth rate. In addition, it is not clear how different bacteria use energy sources in the process of transferring genetic material through conjugation. Therefore, it seems likely that nutrient concentration does not independently drive conjugation efficiency, suggesting other factors, such as growth rate under the influence of nutrient concentration, impact conjugation frequency.

Similar to previous part, the number of transconjugants were compared for two nutrient levels grown under the same growth rate during the course of experiments (Figure 3.5).

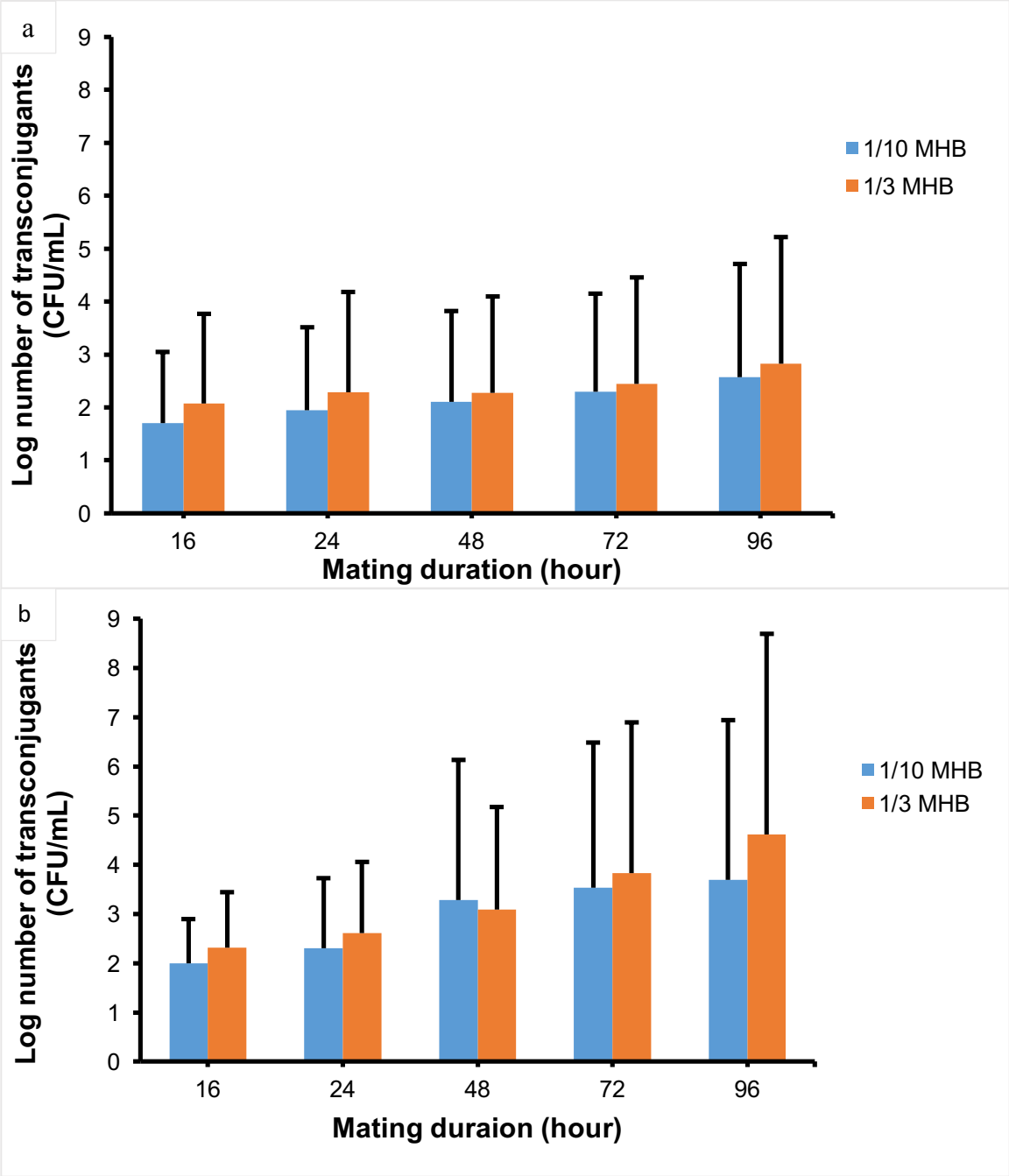


Figure 3.5. The effect of nutrient level on number of transconjugants for 1/3 MHB and 1/10 MHB nutrient level at a) 0.45 hr^{-1} and b) 0.15 hr^{-1} growth rate. Errors bars are from six replicate chemostat reactors.

MANOVA exhibited no significant interaction effect on number of transconjugants for different nutrient backgrounds in different growth conditions.

The nutrient concentration defines the cell density in the chemostat reactor (Ziv, N., Brandt, N. J., Gresham, 2013). Therefore, higher nutrient level led to higher cell density. Since the number of transconjugants can be the summation of conjugation event and subsequent growth of transconjugants, the slight higher number of transconjugants in higher nutrient availability was due to the higher cell density of transconjugants.

3.4 Minimum Inhibitory Concentration Change

To determine the effect of the plasmid transfer from donor to recipient, minimum inhibitory concentration (MIC) for tetracycline were measured under different growth conditions (Figure 3.6). The MIC test showed the immediate increase in resistance level for transconjugants probably due to plasmid transfer. The MIC of transconjugants increased from 2 to 128 mg/L and 2 to 64mg/L for high and low growth rate, respectively, regardless of nutrient level.

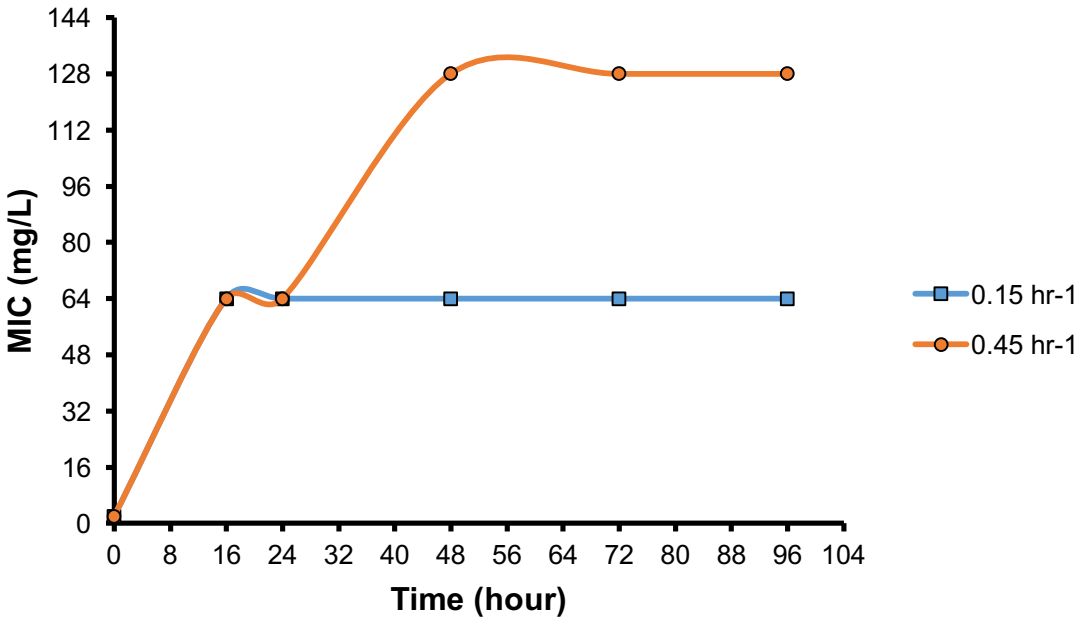


Figure 3.6. Change in MIC relative to J53 control for tetracycline, following 4 days of conjugation experiment under 0.45 hr^{-1} and 0.15 hr^{-1} growth rate for either 1/10 MHB or 1/3 MHB in the reactor.

Since different nutrient background had the similar effect on increasing MIC value for transconjugants, the transconjugants' MIC were influenced by growth rate, being 2-fold higher at 0.45 hr^{-1} than at 0.15 hr^{-1}. Comparably, the MIC for the donor increased from 2 to 256 mg/L after receiving plasmid from WWTP in the filter mating experiment. As observed, the change in MIC in transconjugants was unexpectedly lower than donor although they had the same plasmid, 2-fold in high growth rate and 4-fold in low growth rate. The increase in MIC in transconjugants could not be because of mutation as MIC changed in mutation experiment was insignificant, 2 to 4 mg/L. To check the presence of the plasmid in recipients and explain why the MIC of transconjugants and original donor is not exactly the same, the genomic of donor, recipient and transconjugants were studied.

3.5 Effect of Growth Rate on Mutation

Both high and low growth rate resulted in MIC ranging between 2 and 4 mg/L for 1/3 MHB nutrient level after 96 hours of the experiment. The only exception was the higher growth rate in lower nutrient level, which showed an increase MIC from 2 to 8 mg/L (Figure 3.7).

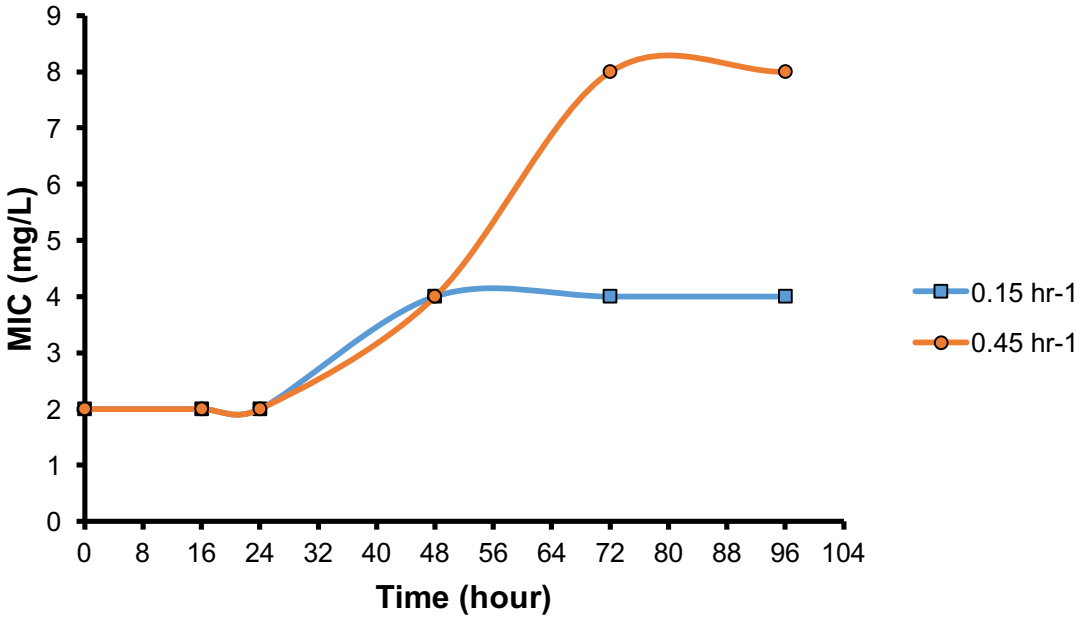


Figure 3.7. Change in MIC relative to J53 control for tetracycline, following 4 days of mutation experiment under 0.45 hr^{-1} and 0.15 hr^{-1} growth rate for 1/10 MHB in the reactor.

For decades, it has been believed that bacteria growing faster are typically more susceptible to antibiotics than bacteria that are slow growing [101]. However, our results showed that the susceptibility of a culture is likely independent of growth rate, at least for a short time. A simple explanation is that most of the studies have been conducted in the batch reactors, meaning the term growth rate has been basically used for the metabolic state rather than growth rate. In this context, the correct statement will be susceptibility is

less notable for stationary phase cells compared to exponentially growing. In the current experimental set-up, MIC was measured during the exponential growth phase, for the two growth rates. In this case, having cells in their healthiest stage of growth, the number of generation, nutrient availability, and the tetracycline stressor can affect either the emergence or proliferation of resistant colonies, denoting that even growth rate cannot be exclusively considered as mutation-driven factor. Therefore, the slight increase in MIC for higher growth rate/low nutrient availability toward the end of the experiments cannot be related only to growth rate. In addition, the culture-based MIC measurement reports the result based on the most resistance cells within the inoculation [102]. Therefore, emergence of limited mutants could increase the overall MIC of the culture. Existing resistance colonies can be either because of their preexisting in the culture or better fitness cost compared to susceptible colonies in the more stressful condition (i.e., higher growth rate/low nutrient level).

3.6 Effect of nutrient background on mutation

The effect of nutrient availability on vertical gene transfer (i.e., mutation) was insignificant in the course of experiments (Figure 3.8). Although a bit increases in MIC was only observed for higher growth rate toward the end of experiments, the change in MIC for both nutrient backgrounds was the same.

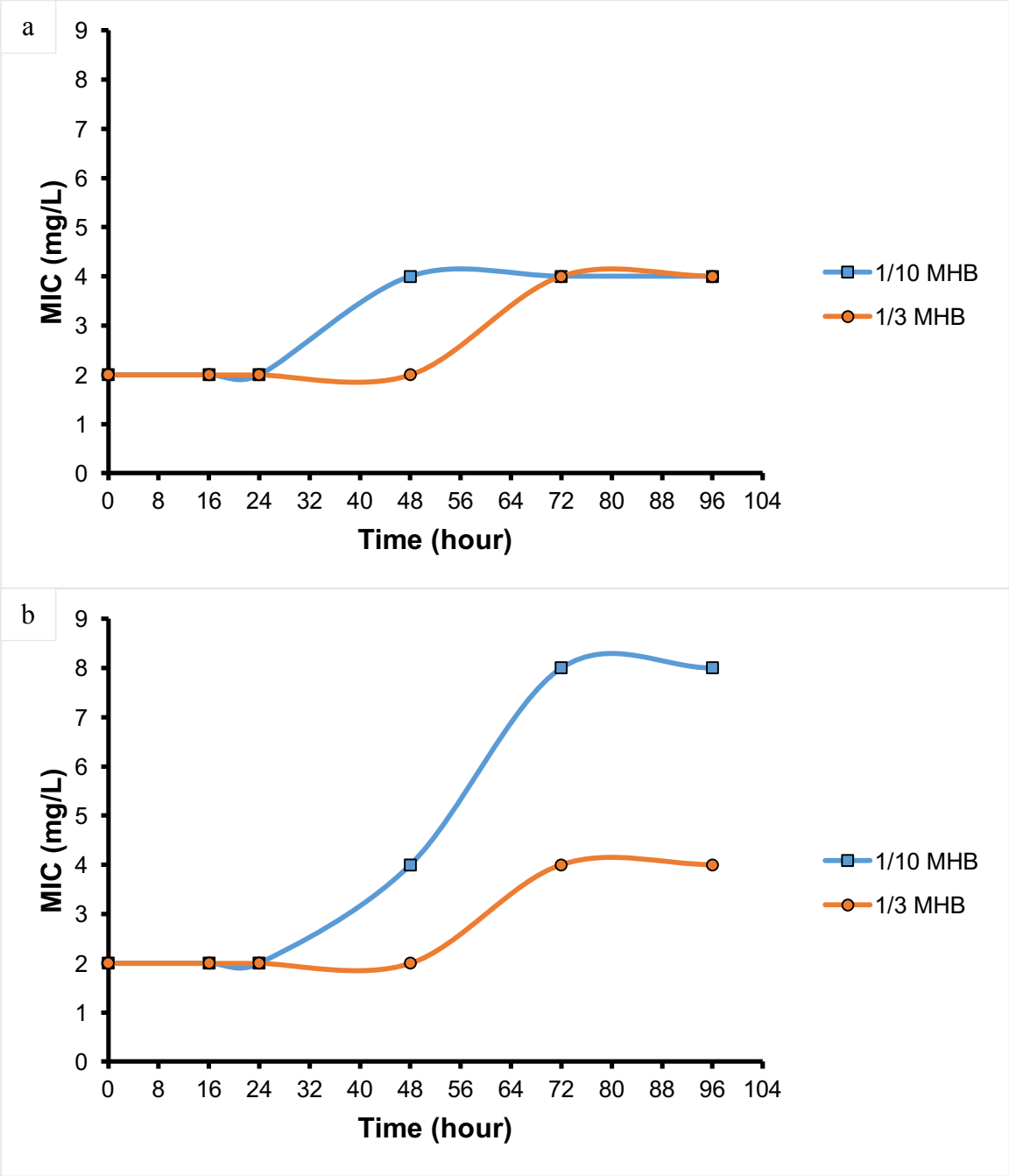


Figure 3.8. Change in MIC relative to J53 control for tetracycline, following 4 days of mutation experiment for 1/10 MHB and 1/3 MHB under a) 0.45 hr^{-1} and b) 0.15 hr^{-1} growth rate in the reactor.

For mutation experiments, the pure culture of J53 was inoculated in the reactors.

As we used continuous reactors, the nutrient concentration solely defined the cell density

and did not affect growth rate. Since the same effect on MIC was observed in conjugation experiments for higher growth rate, we can conclude that generation time or growth rate is a key term for mutation rather than nutrient concentration. Following these results, long-term evolution experiments reported that the genome is highly dynamic and evolving genomes become increasingly different in descendants from ancestor, increasing the probability of emerging beneficial mutations and consequently change in phenotypic characteristics [103].

4.7 Whole Genome Sequencing

To confirm the transfer of tetracycline resistant plasmids from the donor cells to the recipient cells, whole genome sequencing was conducted to compare the genome of donor, recipient, and transconjugants. The sequence of all possible plasmids was examined in the donor to identify tetracycline resistance genes (Table C2). Out of the seven plasmids identified, the CW_60 plasmid was the only plasmid that contained known tetracycline resistant genes (i.e., *tetA* and *tetR*). Figure 3.9 shows the fully assembled the CW_60 plasmid.

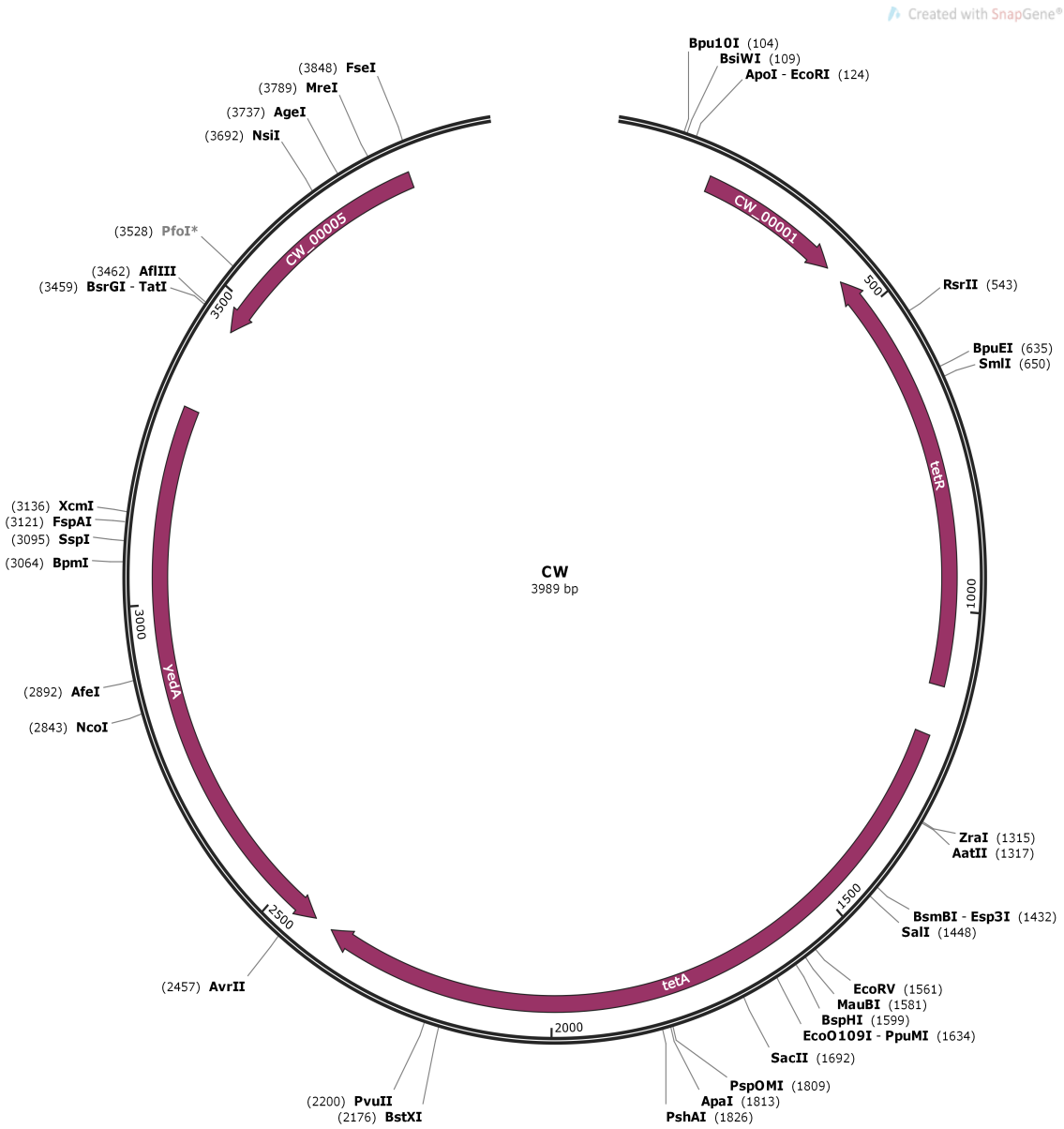


Figure 3.9. The sequenced plasmid in donor annotated for the tetracycline resistance and other known genes.

The sequence of CW_60 will be submitted to GenBank. The sequence of the CW_60 plasmid was searched against databases to check if it matches any known plasmid. The CW_60 plasmid shared more than 98% nucleotide sequence identity with plasmids in *E. coli*, *Salmonella enterica*, *Klebsiella pneumonia*, *Shigella flexneri* and

Shewanella algae strains. Table 3.1 shows four plasmid entries, out of all BLAST hits, containing sequences similar to the CW_60 plasmid.

Table 3.1 Identical plasmids to CW_60 in other studies.

Description	CW_60 Coverages	Resistances[b]	Reference
Uncultured bacterium IncP-1beta multiresistance plasmid pB10	100 %	Mer^r Sm^r Te^r	[104]
Salmonella enterica subsp. Tetracycline and ampicillin resistance transposons Tn1721-like an Tn3-like in plasmid pUO-SoR1	100 %	Su^r Sm^r Te^r SxT^r AP^r	[105]
Escherichia coli strain 2009-52 plasmid psDJ2009-52F	100 %	AP^r SxT^r Te^r Sm^r	[106]
Escherichia coli strain MRSN352231 plasmid pMR0716_tem1	100 %	Amc^r Cf^r Cl^r En^r Te^r SxT^r Te^r FEP^r CAZ^r IMP^r	[107]

[b]AP, ampicillin; Amc, amoxicillin-clavulanic acid; Cf, cephalothin; Cl, colistin; Sm, streptomycin; Su, sulfonamides; Te, tetracycline; SxT, trimethoprim-sulfamethoxazole.

For example, plasmid pB10 was isolated from a wastewater treatment plant in Germany and mediated resistance to the antimicrobial agents tetracycline, amoxicillin, streptomycin, and sulfonamides [104]. They mentioned that the plasmid may rapidly spread among bacterial populations due to the transposons and integrons on it. In another study, a similar plasmid was found in 54 isolates of the foodborne pathogen *Salmonella enterica* serovar Ohio [105]. Two large conjugative plasmids similar to CW_60 contained ARGs

corresponding to tetracycline, streptomycin, trimethoprim, sulphonamides, ampicillin, gentamicin, kanamycin, and chloramphenicol. Lastly, plasmid psDJ2009-52F, which shared 99 percent identity with CW_60, was recovered from two *E. coli* strains isolated from a patient in Sydney Adventist Hospital (Sydney, NSW, Australia). The *E. coli* strains containing psDJ2009-52F showed reduced susceptibility to ampicillin, trimethoprim, sulfamethazole, tetracycline, and streptomycin [106].

The size of CW_60 compared to similar plasmids in databases indicated that CW_60 is likely part of a megaplasmid, which is prevalent in the environment. Moreover, the presence of similar plasmids in different bacteria genre demonstrated that the plasmid can be easily transferred between different species via horizontal gene transfer. Here, CW_60 was completely transferred to recipient grown under 0.45 hr^{-1} growth rate (i.e., T45), and only was 8 bases shorter in transconjugants (Figure 3.10).

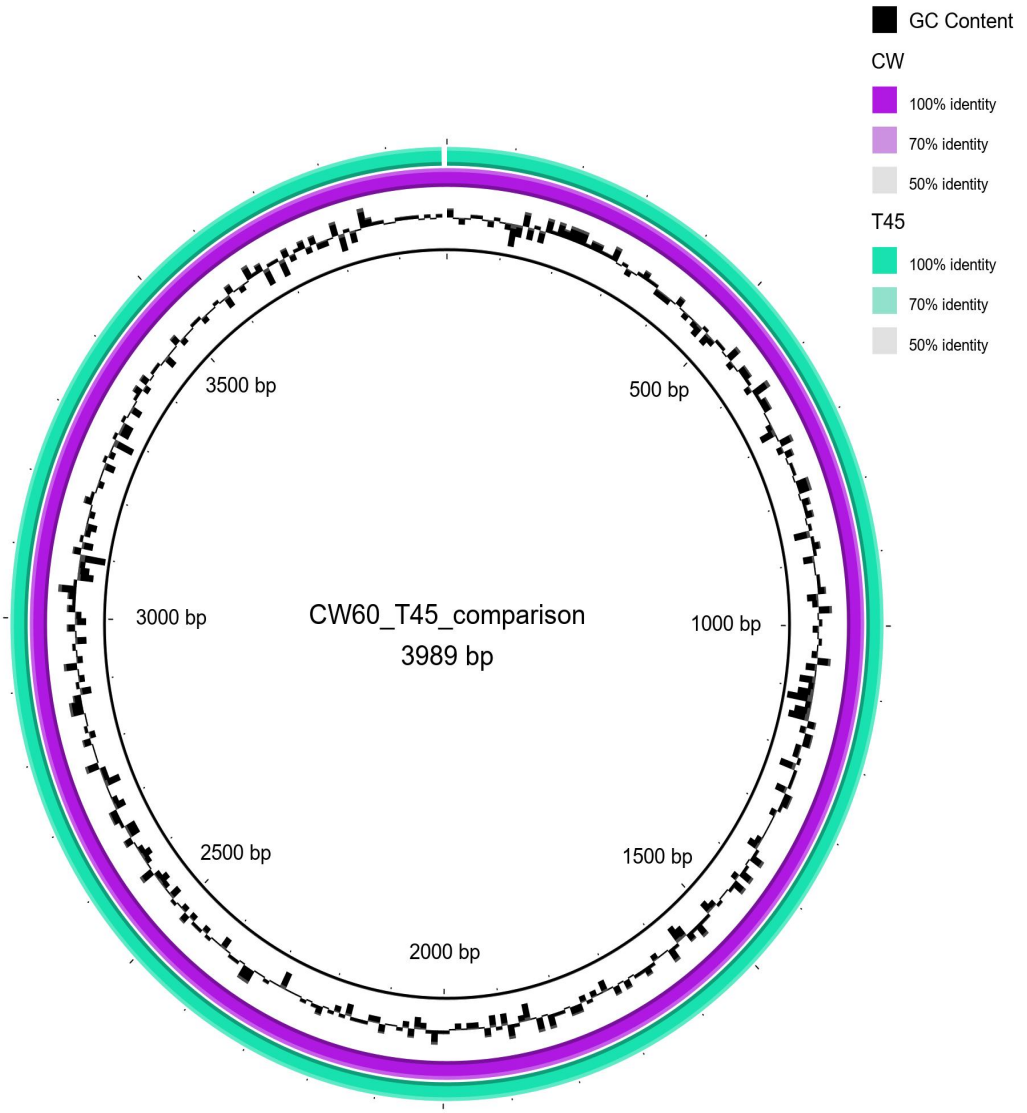


Figure 3.10. Comparison of CW60 plasmid in donor (i.e., purple circle) and transconjugants (i.e., green circle) grown at 0.45 hr^{-1}. The small open area on the top of green circle confers the plasmid in transconjugants were 8 bases shorter than the plasmid in donor.

We could not find CW_60 in transconjugants grown under the growth rate of 0.15 hr^{-1} (i.e., T15). However, other plasmids that were present in donor and T45 were found in T15 (data not shown). The absence of CW_60 in T15 could be due to fragmentation.

These results proved that a mobilizable plasmid containing tetracycline resistance genes were transferred from WWTP effluent to donor, and then later from donor to recipient.

The second question that we tried to answer was the discrepancy in MIC level between donor and transconjugants grown under high and low growth rate. The CW_60 was the reason for the increase in MIC in donor and T45. Comparably, there was not any particular evidence to explain lower MIC in T15 compared to donor and T45. In addition, genomic study is not sufficient to answer this question as this difference in MIC can probably due to the experimental condition, heterogeneity of MIC of colonies in the culture, and compatibility of plasmid [108]. Therefore, further studies needed to assess wide variety of possible parameters and reveal the mystery.

To check mutation events, genome of the recipient grown under different growth rate were assembled (i.e., M45 and M15 for mutants grown under 0.45 hr^{-1} and 0.15 hr^{-1} growth rate, respectively). There were only three mutation events in M15 compared to original recipient (Table 3.2).

Table 3.2 Mutations in recipient grown at 0.45 hr^{-1} and 0.15 hr^{-1}.

Sample	Gene Symbol	Mutation	Description
M45	yaaA	A E 73; D A 242;	peroxide stress resistance protein YaaA
	cbrA	T A 243; T A 292; V M 345;	colicin M resistance protein
	yfgI	L F 5; I V 54;	nalidixic acid resistance protein YfgI
	tehA	A T 60;	tellurite resistance protein
	rclC	W R 28;	reactive chlorine species resistance protein C
	hslJ	A P 14; N I 94; L V 134;	lipoprotein implicated in Novobiocin resistance
M15	nohD	E * 82; E Q 84;	DLP12 prophage%3B putative DNA-packaging protein NohD
	insH-5	I V 83; A P 117; K E 185; G S 187; H N 190;	Rac prophage%3B IS5 transposase and trans-activator
	pinQ	E G 38; K * 82; D A 194;	Qin prophage%3B putative site-specific recombinase

The first mutation in T15 was found in nohD gene. This gene is typically near a site of targeted chromosome cleavage by lambda terminase that introduces double-strand cleavages in DNA [109]. Therefore, the mutation happened in nohD is part of DNA recombination and there was no beneficial function for recipient to combat tetracycline. The second mutation in T15 was on insH-5 gene. InsH-5 interacts with the termini of the IS5 sequence [110]. The IS5 can enhance gene transcription when it is placed on either side of the promoter for a target gene [111]. Hence, the interaction between insH-5 and the IS5 sequence plays a key role in transcription enhancement. The exact function of insH-5 in combating antibiotic resistance is not clear. The third mutation in recipient grown under the lower growth rate was on the pinQ gene. There was no information about the involvement of pinQ in antibiotic resistance. We concluded that all mutations happened in recipient grown at 0.15 hr^{-1} was not related to antibiotic resistant capability. The

phenotypical characteristics of recipient and M15 confirmed our conclusion as they showed similar MIC level.

On the other hand, the MIC for M45 was 8 mg/L which was 4 times higher than that for the original recipient. Genomic results showed six mutations on the M45 genome (Table 3.2). Between those mutations, yaaA was peroxide stress resistance protein, which may play a role in tetracycline resistance. This mutation likely happened due to a stress response of recipient in reaction to tetracycline presence, a process known as stringent response [112]. It showed that tetracycline produces lethal oxidative stress to kill the bacteria [113]. This means for cells facing oxidative stress induced by tetracycline, they produced antioxidant enzymes to survive [114]. In addition, yaaA prevents oxidative damage to both DNA and proteins by diminishing the amount of unincorporated iron within the cell [115]. The higher MIC for recipient grown at 0.45 hr^{-1} compared to recipient may also be attributed to the mutation in the yaaA gene.

CHAPTER 4. CONCLUSIONS AND FUTURE WORK

4.1 Conclusions

Wastewater treatment plants are often considered as hotspots for ARGs and mobile genetic elements. The treatment processes are not able to completely remove antibiotics or ARGs from wastewater. Therefore, the wastewater effluent from WWTPs often contains a wide variety of antibiotics and ARGs, which can subsequently cause the proliferation of ARGs in receiving water bodies. Although the role of the environment in spreading ARGs has been recognized, our understanding of this risk is still limited. Here, the effects of nutrient level and growth rate on the vertical and horizontal transfer of ARGs in surface water receiving wastewater effluent were investigated. From this research, the following conclusions can be made:

- Higher growth rate exhibited higher conjugation frequency than lower growth rate.
- Nutrient level had not significantly impact on conjugation frequency under different growth conditions.
- The number of transconjugants in the reactors is correlated to generation numbers in the reactor.
- The resistance level was higher for cells receiving ARG-bearing plasmids through HGT than cells acquiring ARGs through mutation.
- Whole genome sequencing confirmed transfer of ARG-bearing plasmid between *E. coli* cells.

4.2 Future Work

Recently, mathematical models have been introduced as a simple tool to enhance our understanding of the emergence, spread, and persistence of ARGs in the environment [116–118]. These mathematical models can lead us to find knowledge gaps and identify parameters and processes that are important in estimate the risk of ARGs in the environment. One advantages of modelling is that once calibrated models can be used to simulate different environmental conditions, which would be expensive to test in the lab. Mathematical models for plasmid transfer in batch systems, biofilm, and solid surface have successfully been explored [119–121]. For example, the 'end point' approach by Simonsen et al. is one of the most popular models in plasmid transfer in a batch system [122]. However, due to the complexity in operation, continuous cultures (chemostat) has not been used for plasmid transfer experiment, let alone being modelled. However, the mathematic principal of chemostat reactor has been extensively studied and well-understood. Hence, this experimental design is a promising approach in order to better understand the circumstance in the environment using data acquired from chemostat reactors. Then, by establishing a model calibrated using the data from the chemostat reactors, we will be able to predict the proliferation of ARGs in surface water under other environmental conditions.

REFERENCES

1. Bennett JW, Chung K-T. Alexander Fleming and the Story of Penicillin. *Adv Appl Microbiol*. 2001;49:56. https://pdf.sciencedirectassets.com/273037/1-s2.0-S0065216400X00044/1-s2.0-S0065216401490137/main.pdf?x-amz-security-token=AgoJb3JpZ2luX2VjEGQaCXVzLWVhc3QtMSJHMEUCIQDBxZ0COi3w34Q623NXCYjBol%2FCsLoYCOcXo7gbc6Yr1QIgMqHJFT5Ag9hsCL6t89o6kOQavjw0xHs7Js6SF6mOfk.
2. Alexander F. Penicillin. *Br Med J*. 1941;2:386.
3. Fischbach MA, Walsh CT. Antibiotics for Emerging Infections. *Science (80-)*. 2009;325(5944):1089-1093. doi:10.1126/science.1176667.Antibiotics.
4. Waves P, Media L. Antimicrobial resistance: global report on surveillance. *Clin Microbiol Rev*. 2013;26:185-230. doi:10.1007/s13312-014-0374-3.
5. Alanis AJ. Resistance to antibiotics: Are we in the post-antibiotic era? *Arch Med Res*. 2005;36(6):697-705. doi:10.1016/j.arcmed.2005.06.009.
6. Houndt T, Ochman H. Long-term shifts in patterns of antibiotic resistance in enteric bacteria. *Appl Environ Microbiol*. 2000;66(12):5406-5409. http://www.ncbi.nlm.nih.gov/pubmed/11097921%0Ahttp://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC92475.
7. Smith DH. R factor infection of Escherichia coli lyophilized in 1946. *J Bacteriol*. 1967;94(6):2071-2072.
8. Dcosta VM, King CE, Kalan L, et al. Antibiotic resistance is ancient. *Nature*. 2011;477(7365):457-461. doi:10.1038/nature10388.

9. Martínez JL, Coque TM, Baquero F. What is a resistance gene ? Ranking risk in resistomes. *Nat Publ Gr*. 2014;13(2):116-123. doi:10.1038/nrmicro3399.

10. Durso LM, Miller DN, Wienhold BJ. Distribution and Quantification of Antibiotic Resistant Genes and Bacteria across Agricultural and Non-Agricultural Metagenomes. *PLoS One*. 2012;11(7).

11. Martínez JL. Antibiotics and Antibiotic Resistance Genes in Natural Environments. *Science (80-)*. 2008;321(July):365-368.

12. Allen HK, Donato J, Wang HH, Cloud-Hansen KA, Davies J, Handelsman J. Call of the wild: antibiotic resistance genes in natural environments. *Nat Rev Microbiol*. 2010;8(4):251-259. doi:10.1038/nrmicro2312.

13. Wright GD. Antibiotic resistance in the environment: A link to the clinic? *Curr Opin Microbiol*. 2010;13(5):589-594. doi:10.1016/j.mib.2010.08.005.

14. Riesenfeld CS, Goodman RM, Handelsman J. Uncultured soil bacteria are a reservoir of new antibiotic resistance genes. *Environ Microbiol*. 2004;6(9):981-989. doi:10.1111/j.1462-2920.2004.00664.x.

15. Berendonk TU, Manaia CM, Merlin C, et al. Tackling antibiotic resistance: The environmental framework. *Nat Rev Microbiol*. 2015;13(5):310-317. doi:10.1038/nrmicro3439.

16. Poirel L, Rodriguez-Martinez J-M, Mammeri H, Liard A, Nordmann, Patrice. Origin of Plasmid-Mediated Quinolone Resistance Determinant QnrA. *Antimicrob Agents Chemother*. 2009;49:1-4. doi:10.1128/AAC.49.8.3523.

17. Sommer MOA, Munck C, Toft-Kehler RV, Andersson DI. Prediction of antibiotic resistance: Time for a new preclinical paradigm? *Nat Rev Microbiol*.

2017;15(11):689-696. doi:10.1038/nrmicro.2017.75.

18. Lerminiaux NA, Cameron ADS. Horizontal transfer of antibiotic resistance genes in clinical environments. *Can J Microbiol*. 2018;65(1):34-44. doi:10.1139/cjm-2018-0275.

19. Thomas CM, Nielsen KM. Mechanisms of, and barriers to, horizontal gene transfer between bacteria. *Nat Rev Microbiol*. 2005;3(9):711-721. doi:10.1038/nrmicro1234.

20. Howard Ochman et al. Lateral gene transfer and the nature of bacterial innovation. *Nature*. 2000;405(6784):299-304. http://adsabs.harvard.edu/abs/2000Natur.405..299O.

21. Frost LS, Leplae R, Summers AO, Toussaint A. Mobile genetic elements: The agents of open source evolution. *Nat Rev Microbiol*. 2005;3(9):722-732. doi:10.1038/nrmicro1235.

22. Huijbers, C. PM, Blaak H, et al. Role of the Environment in the Transmission of Antimicrobial Resistance to Humans: A Review. *Environ Sci Technol*. 2015;49(20).

23. Wozniak RAF, Waldor MK. Integrative and conjugative elements: Mosaic mobile genetic elements enabling dynamic lateral gene flow. *Nat Rev Microbiol*. 2010;8(8):552-563. doi:10.1038/nrmicro2382.

24. Swithers KS, Soucy SM, Gogarten JP. The Role of Reticulate Evolution in Creating Innovation and Complexity. *Int J Evol Biol*. 2012;2012:1-10. doi:10.1155/2012/418964.

25. Bennett PM. Plasmid encoded antibiotic resistance: Acquisition and transfer of

antibiotic resistance genes in bacteria. *Br J Pharmacol*. 2008;153:347-357. doi:10.1038/sj.bjp.0707607.

26. Randall Holmes. Medical Microbiology. In: ; 1996.
27. Heredity. *The Horizontal Gene Pool Ð Bacterial Plasmids and GeneSpread*.; 2000. doi:10.1007/s11200-006-0009-9.
28. Klümper U, Riber L, Dechesne A, et al. Broad host range plasmids can invade an unexpectedly diverse fraction of a soil bacterial community. *ISME J*. 2015;9:934-945. doi:10.1038/ismej.2014.191.
29. Hawkey PM, Jones AM. The changing epidemiology of resistance. *J Antimicrob Chemother*. 2009;64(SUPPL.1). doi:10.1093/jac/dkp256.
30. Mell JC, Redfield RJ. Natural competence and the evolution of DNA uptake specificity. *J Bacteriol*. 2014;196(8):1471-1483. doi:10.1128/JB.01293-13.
31. Jonas DA, Elmadfa I, Engel K, Heller KJ, Kozianowski G. Safety Considerations of DNA in Food. *Ann Nutr Metab*. 2001:235-254.
32. Brock TD. *Biology of Micro-Organisms*.; 2010.
33. Darwin C. *The Origins of Species*.; 1859. doi:10.1126/science.146.3640.51-b.
34. Hallgrímsson B, Hall BK. Variation and Variability: Central Concepts in Biology. In: Elsevier Inc.; 1974:1-7. doi:10.1016/b978-012088777-4/50003-x.
35. Zhao H, Arnold FH. Functional and nonfunctional mutations distinguished by random recombination of homologous genes. *Proc Natl Acad Sci*. 1997;94(15):7997-8000. http://www.ncbi.nlm.nih.gov/pubmed/9223302%0Ahttp://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC21544.

36. Thomas JA, Welch JJ, Lanfear R, Bromham L. A generation time effect on the rate of molecular evolution in invertebrates. *Mol Biol Evol*. 2010;27(5):1173-1180. doi:10.1093/molbev/msq009.

37. Kuoppa K, Forsberg Å, Norqvist A. Construction of a reporter plasmid for screening in vivo promoter activity in Francisella tularensis. *FEMS Microbiol Lett*. 2001;205(1):77-81. doi:10.1016/S0378-1097(01)00446-3.

38. von Wintersdorff CJH, Penders J, van Niekerk JM, et al. Dissemination of Antimicrobial Resistance in Microbial Ecosystems through Horizontal Gene Transfer. *Front Microbiol*. 2016;7(February):173. doi:10.3389/fmicb.2016.00173.

39. Bauda P, Lallement C, Manem J. Plasmid content evaluation of activated sludge. *Water Res*. 1995;29(1):371-374. doi:10.1016/0043-1354(94)E0088-N.

40. M D, A P, Selbitschka W. Phenotypic and molecular characterization of conjugative antibiotic resistance plasmids isolated from bacterial communities of activated sludge. *Mol Gen Genet*. 2000;263(3):471-482.

41. Top EM, Springael D. The role of mobile genetic elements in bacterial adaptation to xenobiotic organic compounds. *Curr Opin Biotechnol*. 2003;14(3):262-269. doi:10.1016/S0958-1669(03)00066-1.

42. Kummer K. Drugs in the environment: emission of drugs, diagnostic aids and disinfectants into wastewater by hospitals in relation to other sources - a review. *Chemosphere*. 2001;45:957-969. doi:10.1016/S0045-6535(01)00144-8.

43. Naidoo S, Olaniran AO. Treated wastewater effluent as a source of microbial pollution of surface water resources. *Int J Environ Res Public Health*. 2013;11(1):249-270. doi:10.3390/ijerph110100249.

44. Krzeminski P, Tomei MC, Karaolia P, et al. Performance of secondary wastewater treatment methods for the removal of contaminants of emerging concern implicated in crop uptake and antibiotic resistance spread: A review. *Sci Total Environ*. 2019;648:1052-1081. doi:10.1016/j.scitotenv.2018.08.130.

45. Spongberg AL, Witter JD. Pharmaceutical compounds in the wastewater process stream in Northwest Ohio. *Sci Total Environ*. 2008;397(1-3):148-157. doi:10.1016/j.scitotenv.2008.02.042.

46. Karthikeyan KG, Meyer MT. Occurrence of antibiotics in wastewater treatment facilities in Wisconsin, USA. *Sci Total Environ*. 2006;361(1-3):196-207. doi:10.1016/j.scitotenv.2005.06.030.

47. Bacte ÂM. Natural conjugative plasmids induce bacterial biofilm development. *Nature*. 2001;412(July):24-27.

48. Moura A, Jové T, Ploy MC, Henriques I, Correia A. Diversity of gene cassette promoters in class 1 integrons from wastewater environments. *Appl Environ Microbiol*. 2012;78(15):5413-5416. doi:10.1128/AEM.00042-12.

49. Norman A, Hansen LH, Sørensen SJ. Conjugative plasmids: Vessels of the communal gene pool. *Philos Trans R Soc B Biol Sci*. 2009;364(1527):2275-2289. doi:10.1098/rstb.2009.0037.

50. Szczepanowski R, Krahn I, Linke B, Goesmann A, Pühler A, Schlüter A. Antibiotic multiresistance plasmid pRSB101 isolated from a wastewater treatment plant is related to plasmids residing in phytopathogenic bacteria and carries eight different resistance determinants including a multidrug transport system. *Microbiology*. 2004;150(11):3613-3630. doi:10.1099/mic.0.27317-0.

51. Szczepanowski R, Braun S, Riedel V, et al. The 120 592 bp IncF plasmid pRSB107 isolated from a sewage-treatment plant encodes nine different antiobiotic-resistance determinants, two iron-acquisition systems and other putative virulence-associated functions. *Microbiology*. 2005;151(4):1095-1111. doi:10.1099/mic.0.27773-0.

52. Baquero F, Martínez JL, Cantón R. Antibiotics and antibiotic resistance in water environments. *Curr Opin Biotechnol*. 2008;19(3):260-265. doi:10.1016/j.copbio.2008.05.006.

53. Baquero F, Negri M. Antibiotic-Selective Environments. 1998;27(Suppl 1):5-11.

54. Tenover, F., & McGowan J. Reasons for the Emergence of Antibiotic Resistance. *Am J Med Sci*. 1996;311(1):9-16.

55. Xiong W, Sun Y, Ding X, Wang M, Zeng Z. Selective pressure of antibiotics on ARGs and bacterial communities in manure-polluted freshwater-sediment microcosms. *Front Microbiol*. 2015;6:1-8. doi:10.3389/fmicb.2015.00194.

56. Witte W. Selective pressure by antibiotic use in livestock. *Int J Antimicrob Agents*. 2000;16:19-24. doi:10.1016/s0924-8579(00)00301-0.

57. Kolpin DW, Skopec M, Meyer MT, Furlong ET, Zaugg SD. Urban contribution of pharmaceuticals and other organic wastewater contaminants to streams during differing flow conditions. *Sci Total Environ*. 2004;328(1-3):119-130. doi:10.1016/j.scitotenv.2004.01.015.

58. Watkinson AJ, Murby EJ, Kolpin DW, Costanzo SD. The occurrence of antibiotics in an urban watershed: From wastewater to drinking water. *Sci Total Environ*. 2009;407(8):2711-2723. doi:10.1016/j.scitotenv.2008.11.059.

59. Hernández F, Sancho J V., Ibáñez M, Guerrero C. Antibiotic residue determination in environmental waters by LC-MS. *TrAC - Trends Anal Chem*. 2007;26(6):466-485. doi:10.1016/j.trac.2007.01.012.

60. Jutkina J, Rutgersson C, Flach CF, Joakim Larsson DG. An assay for determining minimal concentrations of antibiotics that drive horizontal transfer of resistance. *Sci Total Environ*. 2016;548-549:131-138. doi:10.1016/j.scitotenv.2016.01.044.

61. Smets BF, Barkay T. Horizontal Gene Transfer: Perspectives at a Crossroads of Scientific Disciplines. *Nat Rev Microbiol*. 2005;3(September):675-678.

62. Hiltunen T, Virta M, Laine AL. Antibiotic resistance in the wild: an eco-evolutionary perspective. *Philos Trans B*. 2017:1-4. doi:http://dx.doi.org/10.1098/rstb.2016.0039.

63. Elsas JD van, Fry JC, Hirsch P, Molin S. Ecology of plasmid transfer and spread. In: *The Horizontal Gene Pool: Bacterial Plasmids and Gene Spread*.

64. Johnsen AR, Kroer N. Effects of stress and other environmental factors on horizontal plasmid transfer assessed by direct quantification of discrete transfer events. *FEMS Microbiol Ecol*. 2007;59(3):718-728. doi:10.1111/j.1574-6941.2006.00230.x.

65. Van Elsas JD, Bailey MJ. The ecology of transfer of mobile genetic elements. *FEMS Microbiol Ecol*. 2002;42(2):187-197. doi:10.1016/S0168-6496(02)00373-2.

66. Lopatkin AJ, Huang S, Smith RP, et al. Antibiotics as a selective driver for conjugation dynamics. *Nat Microbiol*. 2016;1(6):1-8. doi:10.1038/nmicrobiol.2016.44.

67. Zhang PY, Xu PP, Xia ZJ, Wang J, Xiong J, Li YZ. Combined treatment with the

antibiotics kanamycin and streptomycin promotes the conjugation of Escherichia coli. *FEMS Microbiol Lett*. 2013;348(2):149-156. doi:10.1111/1574-6968.12282.

68. Bellanger X, Guilloteau H, Bonot S, Merlin C. Demonstrating plasmid-based horizontal gene transfer in complex environmental matrices: A practical approach for a critical review. *Sci Total Environ*. 2014;493:872-882. doi:10.1016/j.scitotenv.2014.06.070.

69. Smith MD, Guild WR. Improved method for conjugative transfer by filter mating of Streptococcus pneumoniae. *J Bacteriol*. 1980;144(1):457-459. http://www.ncbi.nlm.nih.gov/pubmed/6998966%0Ahttp://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC294685.

70. Smith HL, Waltman P. *The Theory of the Chemostat: Dynamics of Microbial Competition*. Vol 13. Cambridge university press; 1995.

71. Miller AW, Befort C, Kerr EO, Dunham MJ. Design and Use of Multiplexed Chemostat Arrays. *J Vis Exp*. 2013;(72):1-6. doi:10.3791/50262.

72. Yang S, Carlson K. Evolution of antibiotic occurrence in a river through pristine, urban and agricultural landscapes. *Water Res*. 2003;37(19):4645-4656. doi:10.1016/S0043-1354(03)00399-3.

73. Sczesny S, Nau H, Hamscher G. Residue analysis of tetracyclines and their metabolites in eggs and in the environment by HPLC coupled with a microbiological assay and tandem mass spectrometry. *J Agric Food Chem*. 2003;51(3):697-703. doi:10.1021/jf0258407.

74. Kolpin DW, Furlong ET, Meyer MT, et al. Pharmaceuticals, hormones, and other organic wastewater contaminants in U.S. streams, 1999-2000: A national

reconnaissance. *Environ Sci Technol*. 2002;36(6):1202-1211. doi:10.1021/es011055j.

75. Zuccato E, Calamari D, Natangelo M, Fanelli R. Presence of therapeutic drugs inthe environment. *Lancet*. 2000;355(9217):1790-1791. http://linkinghub.elsevier.com/retrieve/pii/S0140673600022716.

76. Niemeyer D, Smalla K, Heuer H, et al. Exogenous Isolation of Antibiotic Resistance Plasmids from Piggery Manure Slurries Reveals a High Prevalence and Diversity of IncQ-Like Plasmids. *Appl Environ Microbiol*. 2000;66(11):4854-4862.

77. Dang B, Mao D, Xu Y, Luo Y. Conjugative multi-resistant plasmids in Haihe River and their impacts on the abundance and spatial distribution of antibiotic resistance genes. *Water Res*. 2017;111:81-91.

78. Koch C, Epidemiologie E, Wernigerode D-. Characterization of Conjugative R Plasmids Belonging to the New Incompatibility Group IncU. *J Gen Microbiol*. 1981;127:155-160.

79. Moura A, Araújo S, Alves MS, Henriques I, Pereira A, Correia ACM. The contribution of Escherichia coli from human and animal sources to the integron gene pool in coastal waters. *Front Microbiol*. 2014;5:1-15. doi:10.3389/fmicb.2014.00419.

80. Wolters B, Kyselková M, Krögerrecklenfort E, Kreuzig R, Smalla K. Transferable antibiotic resistance plasmids from biogas plant digestates often belong to the IncP-1 ε subgroup. *Front Microbiol*. 2015;5(January):1-11. doi:10.3389/fmicb.2014.00765.

81. Carattoli A, Elena VR. Resistance Plasmid Families in Enterobacteriaceae. *Antimicrob Agents Chemother*. 2009;53(6):2227-2238. doi:10.1128/AAC.01707-08.

82. Heuer H, Kro E, Wellington EMH, Egan S, Elsas JD Van. Gentamicin resistance genes in environmental bacteria: prevalence and transfer. *FEMS Microbiol Ecol*. 2002;42:289-302.

83. Yi H, Cho Y-J, Yong D, Chuna J. Genome Sequence of Escherichia coli J53 , a Reference Strain for. *J Bacteriol*. 2012;194(14):3742-3743. doi:10.1128/JB.00641-12.

84. Pruden A, Pei R, Storteboom H, Carlson KH. Antibiotic Resistance Genes as Emerging Contaminants: Studies in Northern Colorado. *Environ Sci Technol*. 2006;40(23):7445-7450.

85. Arpin C, Raymond N, Gon M, Caumette P, Quentin C. Impact of an Urban Effluent on Antibiotic Resistance of Riverine Enterobacteriaceae and Aeromonas spp . *Appl Environ Microbiol*. 2000;66(1):125-132.

86. Detection of antibiotic resistance and tetracycline resistance genes in Enterobacteriaceae isolated from the Pearl rivers in South China. *Environ Pollut*. 2010;158(6):2101-2109.

87. Kaiser FJ, Ansari M, Braunholz D, et al. Loss-of-function HDAC8 mutations cause a phenotypic spectrum of Cornelia de Lange syndrome-like features , ocular hypertelorism , large fontanelle and X-linked inheritance. *Hum Mol Genet*. 2014;23(11):2888-2900. doi:10.1093/hmg/ddu002.

88. Miller AW, Mitchell E, Befort C, Dunham MJ. Dunham Lab Ministat Manual.

2017:1-79.

89. Cruza IC, RaúlRuiz, AnaCuevas, CarlosRevilla, LuisVielva, La F. Determination of conjugation rates on solid surfaces. *Plasmid*. 2012;67(2):174-182.

90. *Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically ; Approved Standard — Ninth Edition*. Vol 32.; 2012.

91. Smith HL. *The Theory of the Chemostat Dynamics of Microbial Competition*. Cambridge university press; 1995.

92. James TW. CONTINUOUS CULTURE OF MICROORGANISMSl. *Annu Rev Microbiol*. 1960;(5):27-46.

93. Herbert D, Elsworth R, Telling RC. The Continuous Culture of Bacteria; a Theoretical and Experimental Study. *J Gen Microbiol*. 2009;14(3):601-622. doi:10.1099/00221287-14-3-601.

94. Ziv, N., Brandt, N. J., Gresham D. The Use of Chemostats in Microbial Systems Biology. 2013.

95. Godwin D, Slater JH. The Influence of the Growth Environment on the Stability of a Drug Resistance Plasmid in Escherichia coli K12. *J Gen Microbiol*. 1979;111(1):201-210. doi:10.1099/00221287-111-1-201.

96. Dang B, Mao D, Xu Y, Luo Y. Conjugative multi-resistant plasmids in Haihe River and their impacts on the abundance and spatial distribution of antibiotic resistance genes. *Water Res*. 2017;111:81-91. doi:10.1016/j.watres.2016.12.046.

97. Olendzenski L, Gogarten JP. Evolution of genes and organisms: The treeweb of life in light of horizontal gene transfer. *Ann N Y Acad Sci*. 2009;1178:137-145. doi:10.1111/j.1749-6632.2009.04998.x.

98. Dujon B. Group I introns as mobile genetic elements: Facts and mechanistic speculations - a review. *Gene*. 1989;82(1):91-114. doi:10.1016/0378-1119(89)90034-6.

99. Gogarten JP, Hilario E. Inteins, introns, and homing endonucleases: recent revelations about the life cycle of parasitic genetic elements. *BMC Evol Biol*. 2006;94(6). doi:10.1186/1471-2164-12-629.

100. Clarke M, Maddera L, Harris RL, Silverman PM. F-pili dynamics by live-cell imaging. *PNAS*. 2008;105(46):17978-17981.

101. Levin BR, Rozen DE. Non-inherited antibiotic resistance. *Nat Rev Microbiol*. 2006;4:556–562.

102. Kuileab JM, Hayali ASN, B.Koenders B, H.ter B. Variations in MIC value caused by differences in experimental protocol. *J Microbiol Methods*. 2009;79(1):44-47.

103. Maddamsetti R, Hatcher PJ, Green AG, Williams BL, Marks DS, Lenski RE. Core Genes Evolve Rapidly in the Long-Term Evolution Experiment with Escherichia coli. *Genome Biol Evol*. 2017;9(4):1072-1083. doi:10.1093/gbe/evx064.

104. Schlüter A, Heuer H, Szczepanowski R, et al. The 64508 bp IncP-1 β antibiotic multiresistance plasmid pB10 isolated from a waste-water treatment plant provides evidence for recombination between members of different branches of the IncP-1 β group. *Microbiology*. 2003;149(11):3139-3153. doi:10.1099/mic.0.26570-0.

105. N M, I R, R R, C MM, R. RM. Molecular basis and evolution of multiple drug resistance in the foodborne pathogen Salmonella enterica serovar Ohio. *Foodborne Pathog Dis*. 2010;7(2):189-198.

106. McKinnon J, Roy Chowdhury P, Djordjevic SP. Genomic analysis of multidrug-

resistant Escherichia coli ST58 causing urosepsis. *Int J Antimicrob Agents*. 2018;52(3):430-435. doi:10.1016/j.ijantimicag.2018.06.017.

107. Snesrud E, Ong AC, Corey B, et al. Analysis of Serial Isolates of mcr-1- Positive Escherichia coli Reveals a Highly Active ISApl1 Transposon. *Antimicrob Agents Chemother*. 2017;61(5):1-10.

108. Jorgensen JH, Ferraro MJ. Antimicrobial susceptibility testing: a review of general principles and contemporary practices. *Clin Infect Dis*. 2009;49(11):1749-1755. doi:10.1086/647952.

109. Kotani H, Kawamura A, Takahashi A, et al. Site-specific dissection of E.coli chromosome by λ terminase. *Nucleic Acids Res*. 1992;20(13):3357-3360. doi:10.1093/nar/20.13.3357.

110. Engler JA, Bree MP Van. The nucleotide sequence and protein-coding capability of the transposable element IS5. *Gene*. 1981;14:155-163.

111. Schnetz K, Rak B. IS5:A mobile enhancer of transcription in Escherichia coli. *NEC Tech J*. 1992;89:1244-1248.

112. Belenky P, Collins JJ. Anti-oxidant strategies to treat stroke. *Science (80-)*. 2011;334. doi:10.1007/978-3-0348-8297-2_23.

113. Poole K. Bacterial Stress Responses as Determinants of Antimicrobial Resistance. *J Antimicrob Chemother*. 2012;67:2069–2089. doi:10.1002/9781119004813.ch10.

114. Nguyen D, Amruta JD, Francois L, et al. Active starvation responses mediate antibiotic tolerance in biofilms and nutrient-limited bacteria. *Science (80-)*. 2011;334(6058):982-986. doi:10.1126/science.1211037.Active.

115. Liu Y, Bauer SC, Imlay JA. The YaaA protein of the Escherichia coli OxyR

regulon lessens hydrogen peroxide toxicity by diminishing the amount of intracellular unincorporated iron. *J Bacteriol*. 2011;193(9):2186-2196. doi:10.1128/JB.00001-11.

116. Levin BR, Lipsitch M, Perrot V, et al. The population genetics of antibiotic resistance. *Clin Infect Dis*. 1997;24 Suppl 1:S9-16. http://www.ncbi.nlm.nih.gov/pubmed/8994776.

117. Bootsma MCJ, van der Horst MA, Guryeva T, ter Kuile BH, Diekmann O. Modeling Non-inherited Antibiotic Resistance. *Bull Math Biol*. 2012;74(8):1691-1705. doi:10.1007/s11538-012-9731-3.

118. Baker M, Hobman JL, Dodd CER, Ramsden SJ, Stekel DJ. Mathematical modelling of antimicrobial resistance in agricultural waste highlights importance of gene transfer rate. *FEMS Microbiol Ecol*. 2016;92(4):1-10. doi:10.1093/femsec/fiw040.

119. Licht TR, Christensen BB, Krogfelt KA, Molin S. Plasmid transfer in the animal intestine and other dynamic bacterial populations: The role of community structure and environment. *Microbiology*. 1999;145(9):2615-2622. doi:10.1099/00221287-145-9-2615.

120. Krone SM, Lu R, Fox R, Suzuki H, Top EM. Modelling the spatial dynamics of plasmid transfer and persistence. *Microbiology*. 2007;153(8):2803-2816. doi:10.1099/mic.0.2006/004531-0.

121. Zhonga X, Droesch J, Fox R, Topb EM, Kronea SM. On the meaning and estimation of plasmid transfer rates for surface-associated and well-mixed bacterial populations. *J Theor Biol*. 2012;294(2):144-152. doi:10.1038/mp.2011.182.doi.

122. Simonsen L, Gordon DM, Stewart FM, Levin BR. Estimating the rate of plasmid transfer: an end-point method. *J Gen Microbiol*. 1990;136(11):2319-2325. doi:10.1099/00221287-136-11-2319.

APPENDICES

Appendix A. Chemostat Parts and Suppliers

Table A1. Parts and Suppliers

Tubing, connectors, and clamps	Source	Item	Quantity
1/16" x 1/8" silicone tubing (tiny)	VWR	89068-468	50' coil pack
3/32" x 7/32" silicone tubing (small) (3/32" tubing uses 1/8" connectors)	VWR	63009-260	50' coil pack
1/4" x 3/8" silicone tubing (medium)	VWR	63009-279	50' coil pack
1/2" x 5/8" silicone tubing (extra large)	VWR	63009-299	50' coil pack
orange green pump tubing, pre-assembled	Watson-Marlow	978.0038.000	pack of 6
Short Blunt Needle for pump tubing, 24 Gauge x 1/2" Length	Brico Medical Supplies	BN2405	pack of 1000
Male luer connector to 1/16" barb	Cole Parmer	EW-45513-00	pack of 25
reducing connector, PP, 3/32" to 1/16"	Cole Parmer	EW-30621-95	pack of 25
female luer, 1/8" barb, PVDF	Cole Parmer	EW-45512-04	pack of 25
male luer lock, 1/8" barb	Cole Parmer	HV-45503-04	pack of 25
barbed Y connector, 1/8" ID	Cole Parmer	HV-30703-92	pack of 10
male luer slip, 1/8" barb	Cole Parmer	HV-45503-26	pack of 25

reducing connector, PVDF, 1/4" to 1/8"	Cole Parmer	EW-30703-50	pack of 10
Male Inline valved connector, Fits tubing: 1/4 in. I.D., Polypropylene	Fisher	05-112-39	pack of 25
Female Inline valved connector, Fits tubing: 1/4 in. I.D., Polypropylene	Fisher	05-112-37	pack of 5
large tubing clamps, 12-position	VWR	63022-407	pack of 6
medium tubing clamps	VWR	63022-405	pack of 12
small tubing clamps	VWR	63022-403	pack of 1000
Day Pinchcock (metal clamp for tubing)	VWR	21730-001	pack of 10
Assorted zip ties	VWR	21800-000	
Silent Air Pumps	Aquarium Guys.com	212422	each
PTFE filters, 0.45 µ, for air filtration	Fisher	R04SP02500	box of 100
#8 silicone stopper, 3/8 in. hole, for babystat bubblers, for vacuum flasks	Fisher	K953715-0801	
4 port manifold	Cole Parmer	EW-06464-85	
hypodermic needle, 16G, 5 in. length (for effluent line)	Air-Tite Products Co., Inc.		
BD Spinal needles, 18G 6 in. length (for air line)			
hypodermic needle, 20G, 1.5 in. length (for media line)			
Foam Silicone stopper, nonstandard size "2", pink	Cole Parmer	EW-06298-06	pack of 20
Blunt Needles for effluent cork, 20 Gauge x 1-1/2" Length	Air-Tite Products Co., Inc.	NB20112	pack of 1000
100 ml bottles for effluent	VWR	16171-004	case of 48
Foam Silicone stopper, non-standard size 12	VWR	EW-06298-22	
5 L Reservoir bottle with bottom hose outlet	VWR	89001-530	
205S/CA16, 16 channel cartridge pump	Watson-Marlow	020.3716.00A	
6-block dry bath	VWR	12621-120	

Appendix B. Donor and Recipient Information

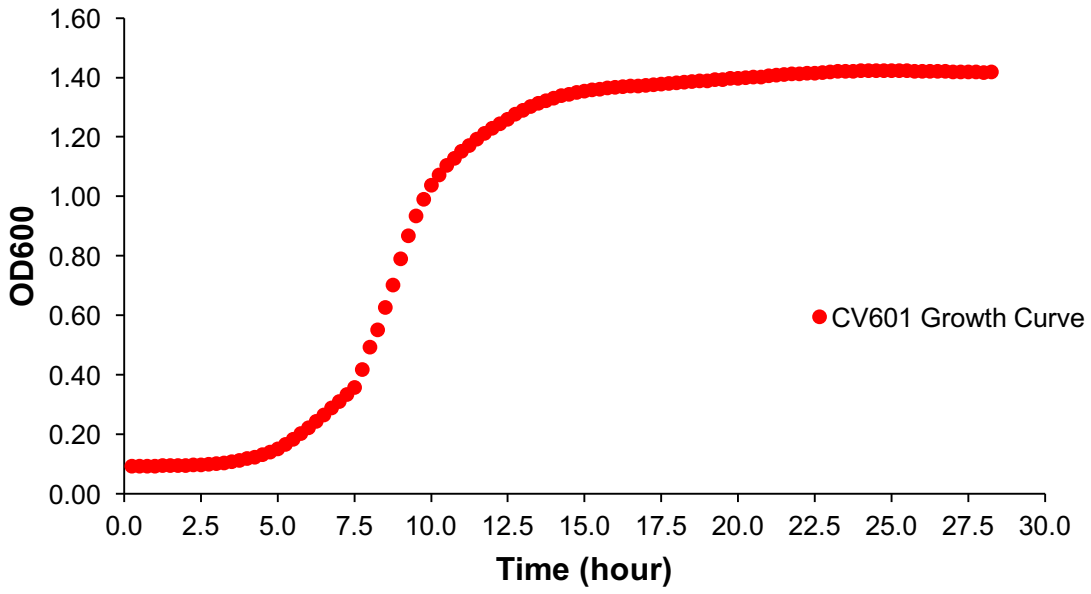


Figure B1. Growth curve of *E. coli* CV601

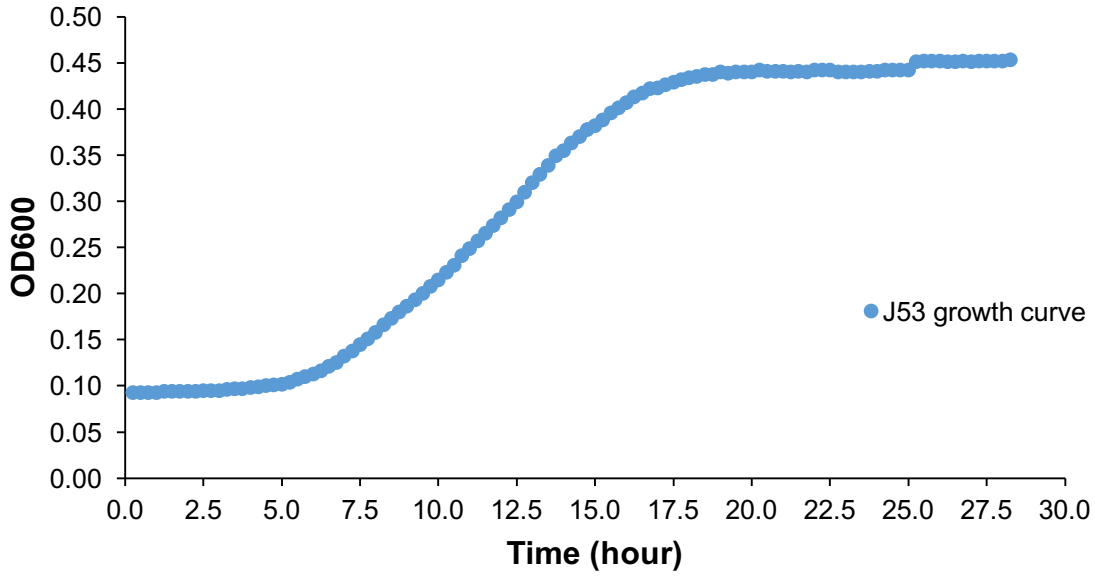
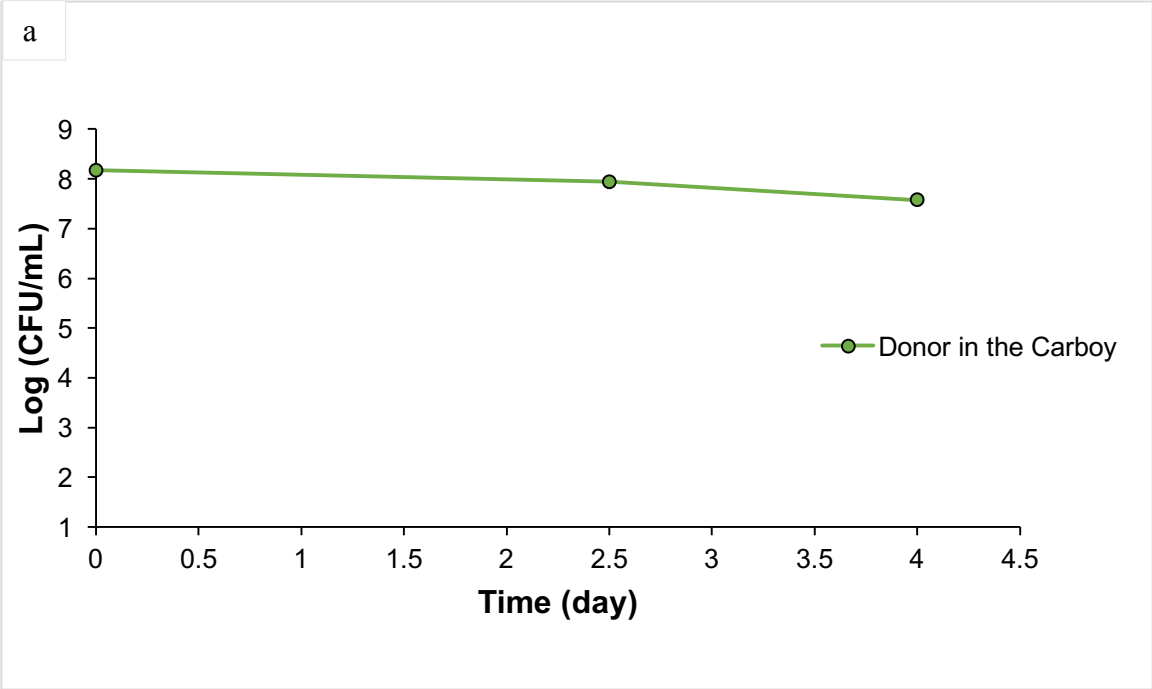


Figure B2. **Growth curve of *E. coli* J53**



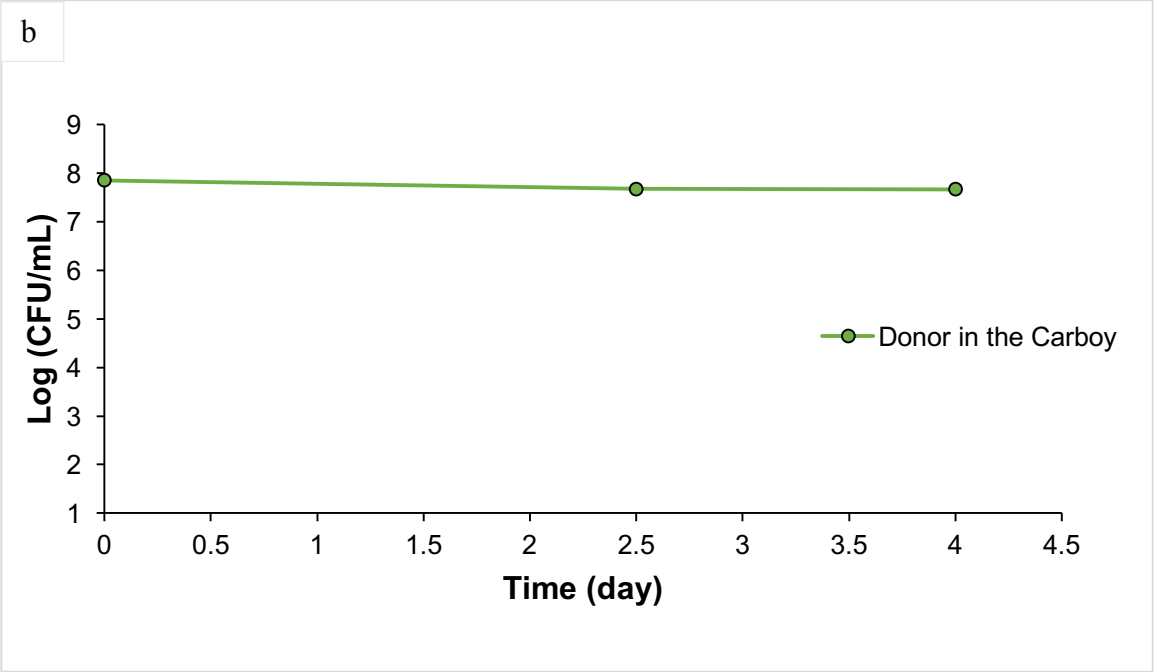


Figure B3. Number of donor in feed reservoir for conjugation experiment grown under a) 0.15 hr^{-1} and b) 0.45 hr^{-1}

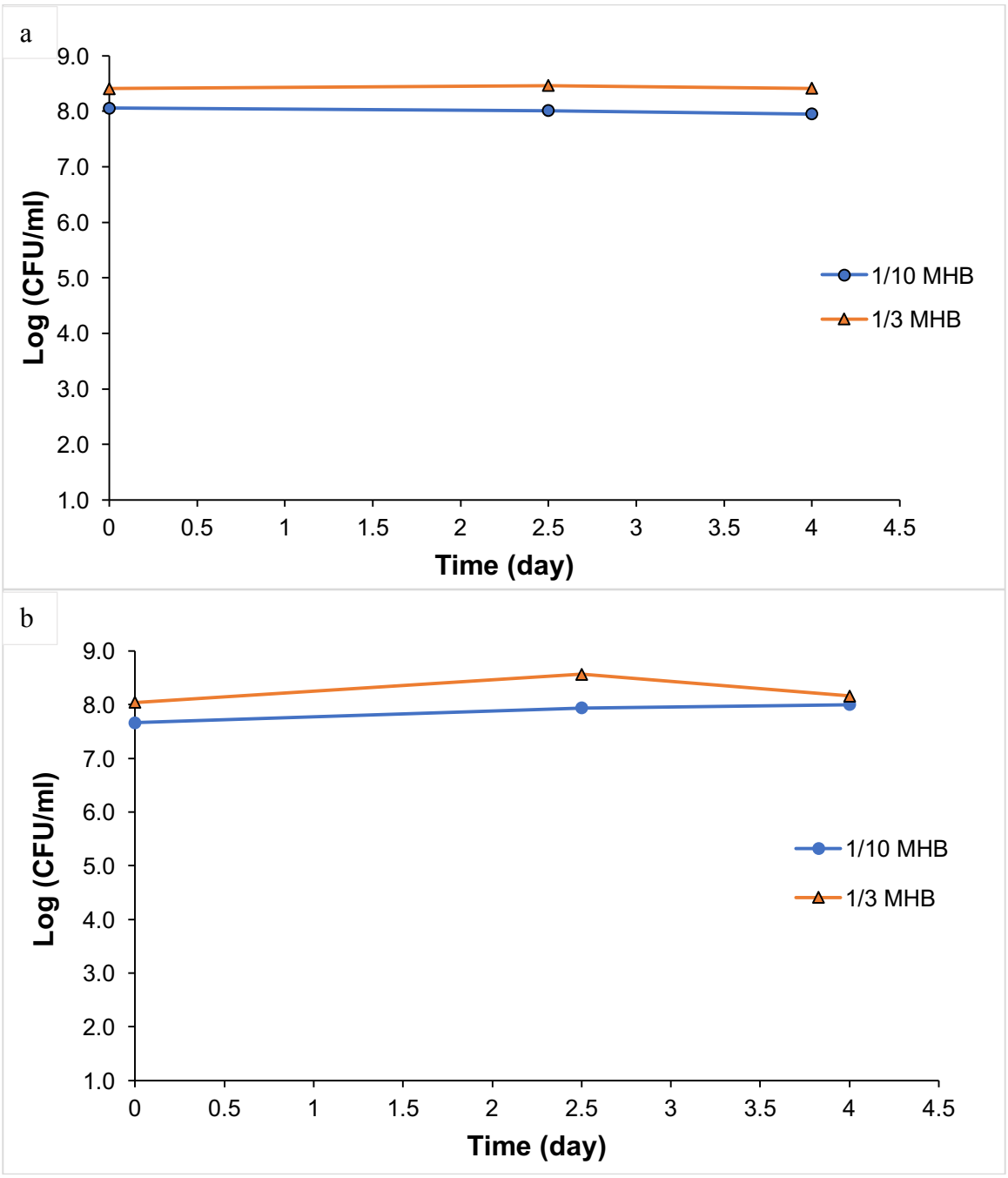


Figure B4. Number of donor in chemostat reactors for two nutrient levels in conjugation experiment grown under a) 0.15 hr^{-1} and b) 0.45 hr^{-1}

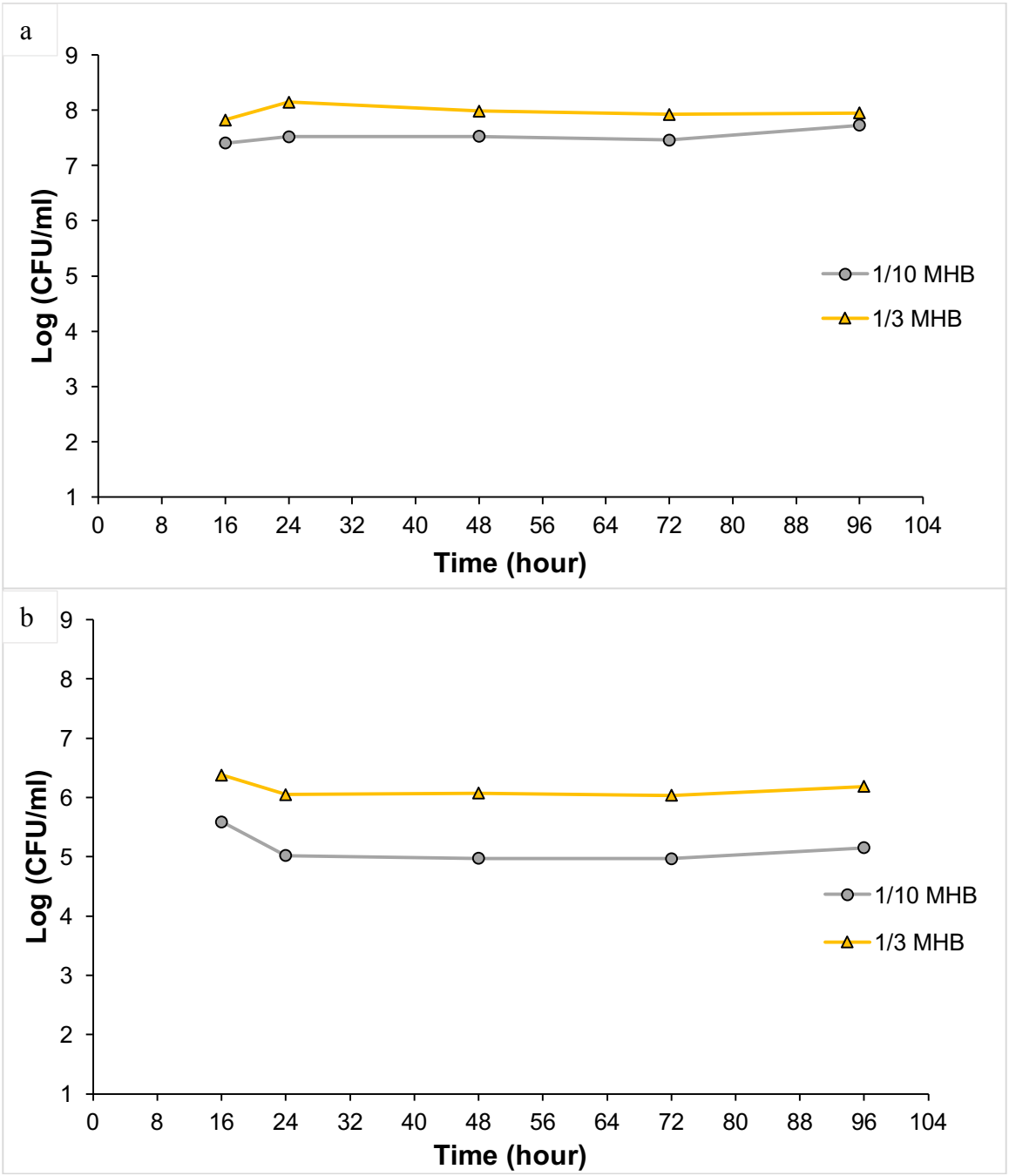


Figure B5. Number of recipient in chemostat reactors for two nutrient levels in conjugation experiment grown under a) 0.15 hr^{-1} and b) 0.45 hr^{-1}

Appendix C. Supporting Information

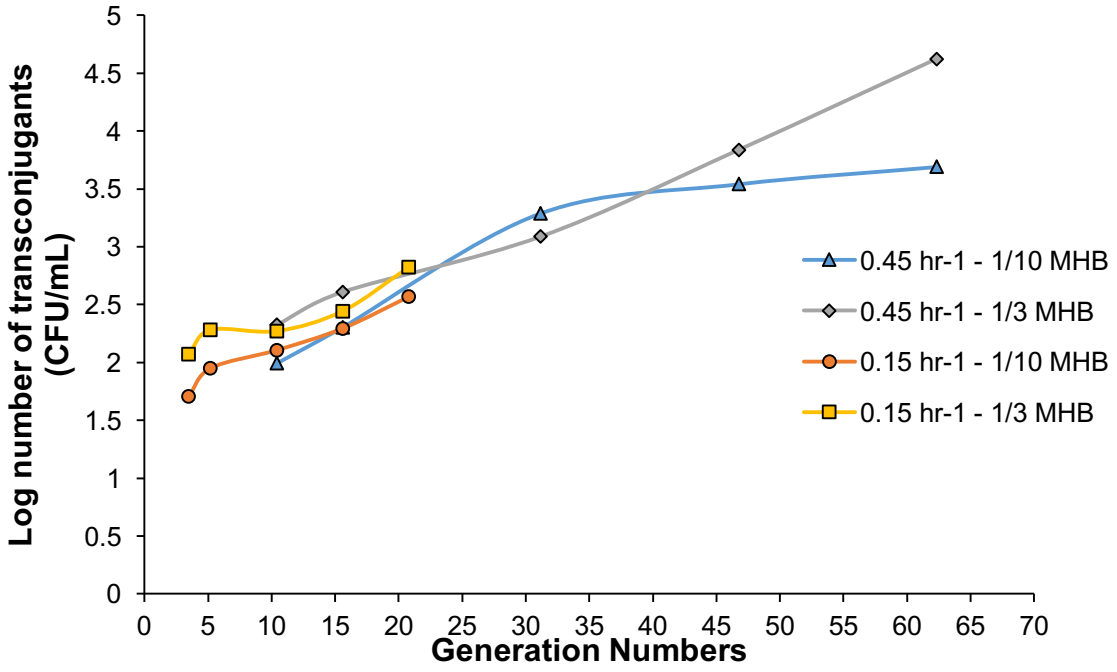


Figure C1. Number of transconjugants in the chemostat reactors for two nutrient levels (i.e., 1/10 MHB and 1/3 MHB) and two growth rates (i.e., 0.15 hr^{-1} and 0.45 hr^{-1}) following the number of generations in conjugation experiment.

Table C2. Possible plasmid presented in donor based on genome content and searching against databases.

ID	length	Gene Content	Is Plasmid? (Blast, PLSDB, PlasmidFinder)
CW_24	84152	49	Y, Y, Y
CW_34	33413	55	Y, Y, Y
CW_47	10104	47	Y, Y, N
CW_56	4636	59	Y, Y, Y
CW_57	4497	60	Y, Y, N
CW_59	4042	43	Y, Y, N
CW_60	3952	62	Y, Y, N
CW_65	2210	53	Y, N, N

Appendix D. Chemostat Protocols

Table D1. A day by day chemostat set-up protocol:

A. Day 1:
   I. Clean all the reactors' part.
   II. Set up the reactors and prepare tubes and needles for them.
   III. Autoclave all bottles and parts.
   IV. Prepare 1 L PBS ➔ Follow the protocol.
   V. Prepare 150ml MHB by adding 3.3gr powder into 150ml DI water.
   VI. Autoclave IV (LIQUID cycle, 121℃, 15min).

B. Day 2:
   I. Prepare 5L of 0.33x Mueller Hinton Broth by adding 36.6gr Mueller H powder into 5000ml ultrapure water.
   II. Prepare 5L of 10x Mueller Hinton Broth by adding 11gr Mueller H powder into 5000ml ultrapure water.
   III. Autoclave I & II (LIQUID cycle, 121℃, 45-50 min).
   IV. Prepare Tetracycline solution ➔ Follow the Protocol.

C. Day 3:
   I. Prepare selective plates (For donor, recipient, and transconjugants). ➔ Follow the Protocol
   II. Prepare MIC plates.
   III. Add 30mg sodium azide to the MHB from Day 1.
   IV. Add a J53 stock culture to the MHB.

D. Day 4:
   I. Wash overnight culture (16hr) with PBS.
   II. Set OD600nm on 0.4 for 10-fold and full MHB reactors, respectively.
   III. Add washed-overnight J53 culture to the reactors. ➔ 15ml.
   IV. Start the pump (10 and 3.5 rpm for 0.45 and 0.15 hr^{-1} dilution rate, respectively)
   V. Measure OD and dilution rate regularly.

E. Day 5:
   I. Prepare 500ml MHB by adding 11gr powder into 500ml DI water (2x).
   II. Autoclave (LIQUID cycle, 121℃, 25 min).
   III. Add kanamycin, rifampicin, and tetracycline to MHB media for donor.
   IV. Store media for donor in the refrigerator.
   V. Prepare selective plates.
   VI. Measure OD and dilution rate.

F. Day 6:
   I. Prepare Tetracycline solution ➔ Follow the Protocol
   II. Prepare selective plates.
   III. Prepare 2L PBS.
   IV. Measure OD & dilution rate.

G. Day 7:
   I. Prepare 2L PBS.
   II. Prepare selective plates.
   III. Prepare MIC plates.
   IV. Measure OD & dilution rate.
   V. Prepare Tet. 2 mg/L by adding 0.1ml of Tet. (256 mg/L) to 12.7ml of water.

H. Day 8:
   I. Prepare selective plates.
   II. Measure OD & dilution rate.
   III. Add a transconjugant CV601 stock culture to the MHB containing Ka, Tet, and Rif.

I. Day 9:
   I. Wash transconjugant CV601 (10hr) by 3L PBS in a sterile caboy.
   II. Add 15 ml of donor to each reactor to have 30ml final volume of each reactor. By doing so, we can keep the dilution ratio constant.
   III. Add tetracycline to the donor carboys to reach 10 μg/L of tetracycline.
   IV. Put transconjugant CV601 in PBS to the chemostat influent line (2L).

J. Day 10:
   I. Take a 2-3ml sample after 16hr from each reactor.
   II. Make serial dilutions in sterile PBS.
   III. Plate the diluted sample on the selective plate which contains sodium azide (200 mg/L) and Tetracycline (10 mg/L). Consider minimum of three parallel plates of an appropriate dilution for each sample.
   IV. Plate samples on selective plates to control donor and recipient numbers.
   V. Incubate the plates at 35℃, UPSIDE DOWN, 2 days.
   VI. Measure MIC ➔ Follow Protocol.

K. Day 11:
   I. Take a 2-3ml sample after 24hr from each reactor.
   II. Make serial dilutions in sterile PBS.
   III. Plate the diluted sample on the selective plate which contains sodium azide (200 mg/L) and Tetracycline (10 mg/L). Consider minimum of three parallel plates of an appropriate dilution for each sample.
   IV. Plate samples on selective plates to control donor and recipient numbers.
   V. Incubate the plates at 35℃, UPSIDE DOWN, 2 days.
   VI. Measure MIC ➔ Follow Protocol.

L. Day 12:
   I. Take a 2-3ml sample after 48hr from each reactor.
   II. Make serial dilutions in sterile PBS.

III. Plate the diluted sample on the selective plate which contains sodium azide (200 mg/L) and Tetracycline (10 mg/L). Consider minimum of three parallel plates of an appropriate dilution for each sample.
IV. Plate samples on selective plates to control donor and recipient numbers.
V. Incubate the plates at 35℃, UPSIDE DOWN, 2 days.
VI. Measure MIC ➔ Follow Protocol.

M. Day 13:
I. Take a 2-3ml sample after 72hr from each reactor.
II. Make serial dilutions in sterile PBS.
III. Plate the diluted sample on the selective plate which contains sodium azide (200 mg/L) and Tetracycline (10 mg/L). Consider minimum of three parallel plates of an appropriate dilution for each sample.
IV. Plate samples on selective plates to control donor and recipient numbers.
V. Incubate the plates at 35℃, UPSIDE DOWN, 2 days.
VI. Measure MIC ➔ Follow Protocol.

N. Day 14:
I. Take a 2-3ml sample after 96hr from each reactor.
II. Make serial dilutions in sterile PBS.
III. Plate the diluted sample on the selective plate which contains sodium azide (200 mg/L) and Tetracycline (10 mg/L). Consider minimum of three parallel plates of an appropriate dilution for each sample.
IV. Plate samples on selective plates to control donor and recipient numbers.
V. Incubate the plates at 35℃, UPSIDE DOWN, 2 days.
VI. Measure MIC ➔ Follow Protocol.

O. Day 15:
I. Take samples from effluent line and make stock culture in glycerol for future analysis.
II. Autoclave all parts and carboy.
III. Wash all tubs and connector/ use 70 percent ethanol for reactors and carboys.
IV. Autoclave all parts one more time.